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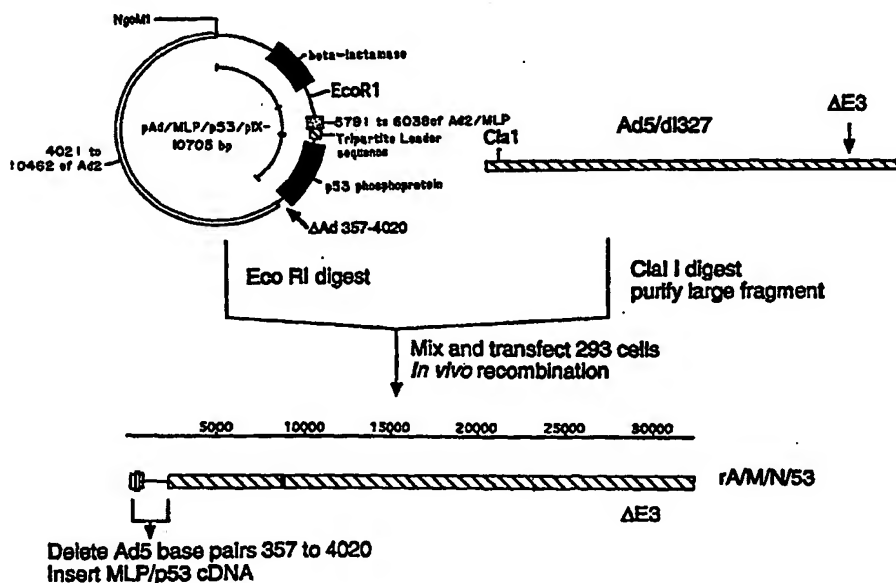
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 15/86, 7/01, 5/10, A61K 48/00, C07K 14/47		A3	(11) International Publication Number: WO 95/11984
			(43) International Publication Date: 4 May 1995 (04.05.95)
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(22) International Filing Date: 25 October 1994 (25.10.94)			
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(71) Applicant: CANJI, INC. [US/US]; Suite 302, 9030 Science Park Road, San Diego, CA 92121 (US).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors: GREGORY, Richard, J.; 4789 Gateshead Road, Carlsbad, CA 92008 (US). WILLS, Ken, N.; 821 Bluffcrest Lane, Encinitas, CA 92024 (US). MANEVAL, Daniel, C.; 12578 Cavallo Street, San Diego, CA 92130 (US).		(88) Date of publication of the international search report: 6 July 1995 (06.07.95)	
(74) Agents: STEINHARDT, Paul, C. et al.; Campbell and Flores, Suite 700, 4370 La Jolla Village Drive, San Diego, CA 92122 (US).			

(54) Title: RECOMBINANT ADENOVIRAL VECTOR AND METHODS OF USE



(57) Abstract

This invention provides a recombinant adenovirus expression vector characterized by the partial or total deletion of the adenoviral protein IX DNA and having a gene encoding a foreign protein or a functional fragment or mutant thereof. Transformed host cells and a method of producing recombinant proteins and gene therapy also are included within the scope of this invention. Thus, for example, the adenoviral vector of this invention can contain a foreign gene for the expression of a protein effective in regulating the cell cycle, such as p53, Rb, or mitotin, or in inducing cell death, such as the conditional suicide gene thymidine kinase. (The latter must be used in conjunction with a thymidine kinase metabolite in order to be effective).

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/12235

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/86 C12N7/01 C12N5/10 A61K48/00 C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBO JOURNAL., vol.6, no.6, 1987, EYNSHAM, OXFORD GB pages 1733 - 1740 GHOSH-CHOUDHURY, G. ET AL. 'Protein IX a minor component of the human adenovirus capsid is essential for the packaging of full length genomes'	1-5, 7-10, 13, 14
Y	see the whole document ---	18-31
A	GENE., vol.13, no.4, 1981, AMSTERDAM NL pages 375 - 386 DIJKEMA, R. ET AL. 'The 'gene for polypeptide IX of human adenovirus type 7' see the whole document --- -/--	1

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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'P' document published prior to the international filing date but later than the priority date claimed

'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search

29 May 1995

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INTERNATIONAL SEARCH REPORT

Internat. Application No
PCT/US 94/12235

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GENE., vol.13, no.4, 1981, AMSTERDAM NL pages 387 - 394 ENGLER J. A. 'The nucleotide sequence of poly peptide IX gene of human adenovirus type 3' see the whole document ---	1
Y	JOURNAL OF CELLULAR BIOCHEMISTRY, vol.SUP 0, no.17D, 17 March 1993 page 129 ZATLOUKAL, K. ET AL. 'Receptor-mediated cytokine gene delivery to tumor cells for generation of cancer vaccines' see abstract NZ 522 ---	15-21
P,Y	JOURNAL OF CELLULAR BIOCHEMISTRY, vol.SUP 0, no.18A, 15 January 1994 page 237 GREGORY, R. J. ET AL. 'Tumor suppressor gene therapy of cancer: adenoviral mediated gene transfer of p53 cDNA into human tumor cell lines' see abstract dZ 307 ---	15-21,25
T	WO,A,94 24297 (RHONE POULENC RORER) 27 October 1994 see the whole document ---	1
P,Y	CANCER GENE THERAPY, vol.1, no.2, June 1994 pages 107 - 112 SHEWACH, D.S. ET AL. 'Enhanced cytotoxicity of antiviral drugs mediated by adenovirus directed transfer of the herpes simplex virus thymidine kinase gene in rat glioma cells' see the whole document ---	18-31
Y	FR,A,2 688 514 (CNRS) 17 September 1993 see claim 3 ---	15,16, 18,19, 23-25,31
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol.87, November 1990, WASHINGTON US pages 8746 - 8750 VENKATESH, L. K. ET AL. 'Selective induction of cytotoxicity to human cells expressing human immunodeficiency virus type 1 tat by a conditionally cytotoxic adenovirus vector' see the whole document -----	18,25

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/ 12235

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 16-24, 26-30 and partially 15, as far as it concerns as in vivo method of treatment, are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 94/12235

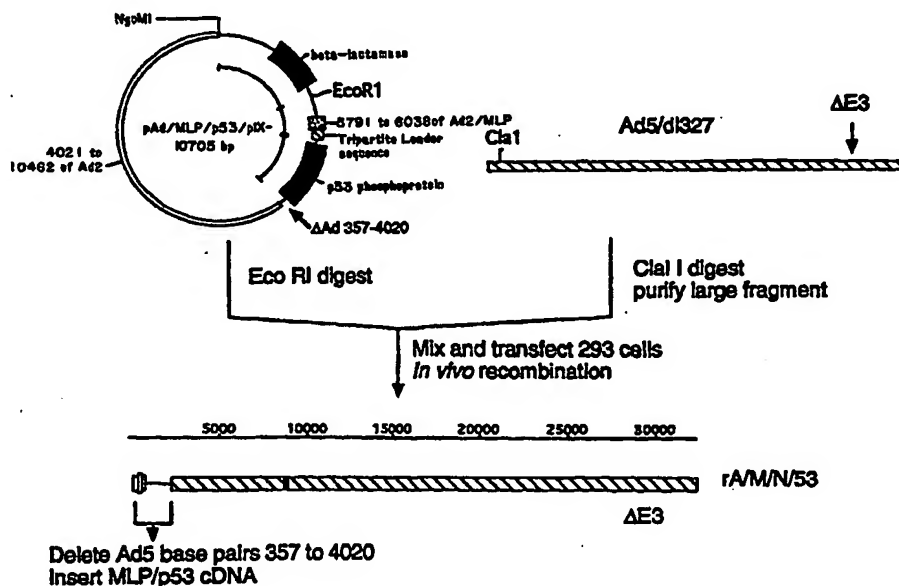
Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9424297	27-10-94	FR-A- 2704234 AU-B- 6572194	28-10-94 08-11-94
FR-A-2688514	17-09-93	AU-B- 3757093 CA-A- 2102302 EP-A- 0593755 WO-A- 9319191 HU-A- 66486 JP-T- 6508039 NO-A- 934061	21-10-93 17-09-93 27-04-94 30-09-93 28-11-94 14-09-94 09-11-93



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			(43) International Publication Date: 4 May 1995 (04.05.95)
(21) International Application Number: PCT/US94/12235		(81) Designated States: AM, AU, BB, BG, BR, BY, CA, CN, CZ, FI, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LV, MD, MG, MN, MW, NO, NZ, PL, RO, RU, SD, SI, SK, TJ, TT, UA, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD, SZ).	
(22) International Filing Date: 25 October 1994 (25.10.94)			
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This invention provides a recombinant adenovirus expression vector characterized by the partial or total deletion of the adenoviral protein IX DNA and having a gene encoding a foreign protein or a functional fragment or mutant thereof. Transformed host cells and a method of producing recombinant proteins and gene therapy also are included within the scope of this invention. Thus, for example, the adenoviral vector of this invention can contain a foreign gene for the expression of a protein effective in regulating the cell cycle, such as p53, Rb, or mitosis, or in inducing cell death, such as the conditional suicide gene thymidine kinase. (The latter must be used in conjunction with a thymidine kinase metabolite in order to be effective).

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RECOMBINANT ADENOVIRAL VECTOR AND METHODS OF USE
BACKGROUND OF THE INVENTION

This application is a continuation-in-part of U.S. Serial No. 08/233,777, filed May 19, 1994, which is a
5 continuation-in-part of U.S. Serial No. 08/142,669 filed October 25, 1993, the contents of which are hereby incorporated by reference into the present disclosure.

Throughout this application, various publications are referred to by citations within parentheses and in the
10 bibliographic description, immediately preceding the claims. The disclosures of these publications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

15 Production of recombinant adenoviruses useful for gene therapy requires the use of a cell line capable of supplying in *trans* the gene products of the viral E1 region which are deleted in these recombinant viruses. At present the only useful cell line available is the 293 cell line
20 originally described by Graham et al. in 1977. 293 cells contain approximately the left hand 12% (4.3 kb) of the adenovirus type 5 genome (Aiello (1979) and Spector (1983)).

Adenoviral vectors currently being tested for
25 gene therapy applications typically are deleted for Ad2 or Ad5 DNA extending from approximately 400 base pairs from the 5' end of the viral genome to approximately 3.3 kb from the 5' end, for a total E1 deletion of 2.9 kb. Therefore, there exists a limited region of homology of approximately
30 1 kb between the DNA sequence of the recombinant virus and the Ad5 DNA within the cell line. This homology defines a region of potential recombination between the viral and cellular adenovirus sequences. Such a recombination results in a phenotypically wild-type virus bearing the Ad5
35 E1 region from the 293 cells. This recombination event

presumably accounts for the frequent detection of wild-type adenovirus in preparations of recombinant virus and has been directly demonstrated to be the cause of wild-type contamination of the Ad2 based recombinant virus Ad2/CFTR-1
5 (Rich et al. (1993)).

Due to the high degree of sequence homology within the type C adenovirus subgroup such recombination is likely to occur if the vector is based on any group C adenovirus (types 1, 2, 5, 6).

10 In small scale production of recombinant adenoviruses, generation of contaminating wild-type virus can be managed by a screening process which discards those preparations of virus found to be contaminated. As the scale of virus production grows to meet expected demand for
15 genetic therapeutics, the likelihood of any single lot being contaminated with a wild-type virus also will rise as well as the difficulty in providing non-contaminated recombinant preparations.

There will be over one million new cases of
20 cancer diagnosed this year, and half that number of cancer-related deaths (American Cancer Society, 1993). p53 mutations are the most common genetic alteration associated with human cancers, occurring in 50-60% of human cancers (Hollstein et al. (1991); Bartek et al. (1991); Levine
25 (1993)). The goal of gene therapy in treating p53 deficient tumors, for example, is to reinstate a normal, functional copy of the wild-type p53 gene so that control of cellular proliferation is restored. p53 plays a central role in cell cycle progression, arresting growth so that
30 repair or apoptosis can occur in response to DNA damage. Wild-type p53 has recently been identified as a necessary component for apoptosis induced by irradiation or treatment with some chemotherapeutic agents (Lowe et al. (1993) A and B). Due to the high prevalence of p53 mutations in human

tumors, it is possible that tumors which have become refractory to chemotherapy and irradiation treatments may have become so due in part to the lack of wild-type p53. By resupplying functional p53 to these tumors, it is
5 reasonable that they now are susceptible to apoptosis normally associated with the DNA damage induced by radiation and chemotherapy.

One of the critical points in successful human tumor suppressor gene therapy is the ability to affect a
10 significant fraction of the cancer cells. The use of retroviral vectors has been largely explored for this purpose in a variety of tumor models. For example, for the treatment of hepatic malignancies, retroviral vectors have been employed with little success because these vectors are
15 not able to achieve the high level of gene transfer required for *in vivo* gene therapy (Huber, B.E. et al., 1991; Caruso M. et al., 1993).

To achieve a more sustained source of virus production, researchers have attempted to overcome the
20 problem associated with low level of gene transfer by direct injection of retroviral packaging cell lines into solid tumors (Caruso, M. et al., 1993; Ezzidine, Z.D. et al., 1991; Culver, K.W. et al., 1992). However, these methods are unsatisfactory for use in human patients
25 because the method is troublesome and induces an inflammatory response against the packaging cell line in the patient. Another disadvantage of retroviral vectors is that they require dividing cells to efficiently integrate and express the recombinant gene of interest (Huber, B.E.
30 1991). Stable integration into an essential host gene can lead to the development or inheritance of pathogenic diseased states.

Recombinant adenoviruses have distinct advantages over retroviral and other gene delivery methods (for

review, see Siegfried (1993)). Adenoviruses have never been shown to induce tumors in humans and have been safely used as live vaccines (Straus (1984)). Replication deficient recombinant adenoviruses can be produced by replacing the E1 region necessary for replication with the target gene. Adenovirus does not integrate into the human genome as a normal consequence of infection, thereby greatly reducing the risk of insertional mutagenesis possible with retrovirus or adeno-associated viral (AAV) vectors. This lack of stable integration also leads to an additional safety feature in that the transferred gene effect will be transient, as the extrachromosomal DNA will be gradually lost with continued division of normal cells. Stable, high titer recombinant adenovirus can be produced at levels not achievable with retrovirus or AAV, allowing enough material to be produced to treat a large patient population. Moreover, adenovirus vectors are capable of highly efficient *in vivo* gene transfer into a broad range of tissue and tumor cell types. For example, others have shown that adenovirus mediated gene delivery has a strong potential for gene therapy for diseases such as cystic fibrosis (Rosenfeld et al. (1992); Rich et al. (1993)) and α_1 -antitrypsin deficiency (Lemarchand et al. (1992)). Although other alternatives for gene delivery, such as cationic liposome/DNA complexes, are also currently being explored, none as yet appear as effective as adenovirus mediated gene delivery.

As with treating p53 deficient tumors, the goal of gene therapy for other tumors is to reinstate control of cellular proliferation. In the case of p53, introduction of a functional gene reinstates cell cycle control allowing for apoptotic cell death induced by therapeutic agents. Similarly, gene therapy is equally applicable to other tumor suppressor genes which can be used either alone or in combination with therapeutic agents to control cell cycle progression of tumor cells and/or induce cell death.

Moreover, genes which do not encode cell cycle regulatory proteins, but directly induce cell death such as suicide genes or, genes which are directly toxic to the cell can be used in gene therapy protocols to directly eliminate the cell cycle progression of tumor cells.

Regardless of which gene is used to reinstate the control of cell cycle progression, the rationale and practical applicability of this approach is identical. Namely, to achieve high efficiencies of gene transfer to express therapeutic quantities of the recombinant product. The choice of which vector to use to enable high efficiency gene transfer with minimal risk to the patient is therefore important to the level of success of the gene therapy treatment.

Thus, there exists a need for vectors and methods which provide high level gene transfer efficiencies and protein expression which provide safe and effective gene therapy treatments. The present invention satisfies this need and provides related advantages as well.

SUMMARY OF THE INVENTION

This invention provides a recombinant adenovirus expression vector characterized by the partial or total deletion of the adenoviral protein IX DNA and having a gene encoding a foreign protein or a functional fragment or mutant thereof. Transformed host cells and a method of producing recombinant proteins and gene therapy also are included within the scope of this invention.

Thus, for example, the adenoviral vector of this invention can contain a foreign gene for the expression of a protein effective in regulating the cell cycle, such as p53, Rb, or mitosin, or in inducing cell death, such as the conditional suicide gene thymidine kinase. (The latter

must be used in conjunction with a thymidine kinase metabolite in order to be effective).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows a recombinant adenoviral vector of this invention. This construct was assembled as shown in Figure 1. The resultant virus bears a 5' deletion of adenoviral sequences extending from nucleotide 356 to 4020 and eliminates the E1a and E1b genes as well as the entire protein IX coding sequence, leaving the polyadenylation site shared by the E1b and pIX genes intact for use in terminating transcription of any desired gene.

Figure 2 shows the amino acid sequence of p110^{RB}.

Figure 3 shows a DNA sequence encoding a retinoblastoma tumor suppressor protein.

Figure 4 shows schematic of recombinant p53/adenovirus constructs within the scope of this invention. The p53 recombinants are based on Ad 5 and have had the E1 region of nucleotides 360-3325 replaced with a 1.4 kb full length p53 cDNA driven by the Ad 2 MLP (A/M/53) or human CMV (A/C/53) promoters followed by the Ad 2 tripartite leader cDNA. The control virus A/M has the same Ad 5 deletions as the A/M/53 virus but lacks the 1.4 kb p53 cDNA insert. The remaining E1b sequence (705 nucleotides) have been deleted to create the protein IX deleted constructs A/M/N/53 and A/C/N/53. These constructs also have a 1.9 kb Xba I deletion within adenovirus type 5 region E3.

Figures 5A and 5B show p53 protein expression in tumor cells infected with A/M/53 and A/C/53. Figure 5A) Saos-2 (osteosarcoma) cells were infected at the indicated multiplicities of infection (MOI) with either the A/M/53 or

A/C/53 purified virus and harvested 24 hours later. The p53 antibody pAb 1801 was used to stain immunoblots of samples loaded at equal total protein concentrations. Equal protein concentration of SW480 cell extracts, which overexpress mutant p53 protein, were used as a marker for p53 size. "O" under the A/C/53 heading indicates a mock infection, containing untreated Saos-2 lysate. Figure 5B) Hep 3B (hepatocellular carcinoma) cells were infected with the A/M/53 or A/C/53 virus at the indicated MOI and analyzed as in part A.) The arrow indicates the position of the p53 protein.

Figures 6A through 6C show p53 dependent Saos-2 morphology change. Subconfluent (1×10^5 cells/10 cm plate) Saos-2 cells were either uninfected (A), infected at an MOI = 50 with (B) the control A/M virus or (C) the A/C/53 virus. The cells were photographed 72 hours post-infection.

Figure 7 shows p53 dependent inhibition of DNA synthesis in human tumor cell lines by A/M/N/53 and A/C/N/53. Nine different tumor cell lines were infected with either control adenovirus A/M (-x-x-), or the p53 expressing A/M/N/53 (-Δ-Δ-), or A/C/N/53 (-O-O-) virus at increasing MOI as indicated. The tumor type and p53 status is noted for each cell line (wt = wild type, null = no protein expressed, mut = mutant protein expressed). DNA synthesis was measured 72 hours post-infection as described below in Experiment No. II. Results are from triplicate measurements at each dose (mean \pm SD), and are plotted as % of media control versus MOI. * H69 cells were only tested with A/M and A/M/N/53 virus.

Figure 8 shows tumorigenicity of p53 infected Saos-2 cells in nude mice. Saos-2 cells were infected with either the control A/M virus or the p53 recombinant A/M/N/53 at MOI = 30. Treated cells were injected

subcutaneously into the flanks of nude mice, and tumor dimensions were measured (as described in Experiment No. II) twice per week for 8 weeks. Results are plotted as tumor size versus days post tumor cell implantation for both control A/M (-x-x-) and A/M/N/53 (-Δ-Δ-) treated cells. Error bars represent the mean tumor size \pm SEM for each group of 4 animals at each time point.

Figure 9 is expression of rAd/p53 RNA in established tumors. H69 (SCLC) cells were injected subcutaneously into nude mice and allowed to develop tumors for 32 days until reaching a size of approximately 25-50 mm³. Mice were randomized and injected peritumorally with 2×10^9 pfu of either control A/C/β-gal or A/C/53 virus. Tumors were excised 2 and 7 days post injection, and polyA RNA was prepared from each tumor sample. RT-PCR was carried out using equal RNA concentrations and primers specific for recombinant p53 message. PCR amplification was for 30 cycles at 94°C 1 min., 55°C 1.5 min., 72°C 2 min., and a 10 min., 72°C final extension period in an Omnigen thermalcycler (Hybaid). The PCR primers used were a 5' Tripartite Leader cDNA (5' - CGCCACCGAGGGACCTGAGCGAGTC-3') and a 3' p53 primer (5' - TTCTGGGAAGGGACAGAAGA-3'). Lanes 1, 2, 4, and 5 are p53 treated samples excised at day 2 or 7 as indicated. Lanes 3 and 6 are from β-gal treated tumors. Lanes 7,8, and 9 are replicates of lanes 4,5, and 6 respectively, amplified with actin primers to verify equal loading. Lane 10 is a positive control using a tripartite/p53 containing plasmid.

Figures 10A and 10B show *in vivo* tumor suppression and increased survival time with A/M/N/53. H69 (SCLC) tumor cells were injected subcutaneously into nude mice and allowed to develop for 2 weeks. Peritumoral injections of either buffer alone (---), control A/M adenovirus (-x-x-), or A/M/N/53 (-Δ-Δ), both viruses (2×10^9 pfu/injection) were administered twice per week for a

total of 8 doses. Tumor dimensions were measured twice per week and tumor volume was estimated as described in Experiment No. II. A) Tumor size is plotted for each virus versus time (days) post inoculation of H69 cells. Error bars indicate the mean tumor size +/- SEM for each group of 5 animals. Arrows indicate days virus injections. B) Mice were monitored for survival and the fraction of mice surviving per group versus time post inoculation of buffer alone (----), control A/M (... ..) or A/M/N/53 (—) virus treated H69 cells is plotted.

Figures 11A through 11C show maps of recombinant plasmid constructions. Plasmids were constructed as detailed in below. Bold lines in the constructs indicate genes of interest while boldface type indicates the restriction sites used to generate the fragments to be ligated together to form the subsequent plasmid as indicated by the arrows. In Figure 11A, the plasmid pACNTK was constructed by subcloning the HSV-TK gene from pMLBKTk (ATCC No. 39369) into the polylinker of a cloning vector, followed by isolation of the TK gene with the desired ends for cloning into the pACN vector. The pACN vector contains adenoviral sequences necessary for *in vivo* recombination to occur to form recombinant adenovirus (see Figure 12). In Figure 11B, the construction of the plasmid pAANTK is shown beginning with PCR amplified fragments encoding the α -fetoprotein enhancer (AFP-E) and promoter (AFP-P) regions subcloned through several steps into a final plasmid where the AFP enhancer and promoter are upstream of the HSV-TK gene followed by adenovirus Type 2 sequences necessary for *in vivo* recombination to occur to form recombinant adenovirus. In Figure 11C, the construction of the plasmid pAANCAT is shown beginning with the isolation of the chloramphenicol acetyltransferase (CAT) gene from a commercially available plasmid and subcloning it into the pAAN plasmid (see above), generating the final plasmid pAANCAT where the AFP enhancer/promoter direct

transcription of the CAT gene in an adenovirus sequence background.

Figure 12 is a schematic map of recombinant adenoviruses ACNTK, AANTK and AANCAT. To construct
5 recombinant adenoviruses from the plasmids described in Figure 11, 4 parts (20 μ g) of either plasmid pACNTK, pAANTK, or pAANCAT were linearized with Eco R1 and cotransfected with 1 part (5 μ g) of the large fragment of
10 Cla 1 digested recombinant adenovirus (rACB-gal) containing an E3 region deletion (Wills et al., 1994). In the resulting viruses, the Ad 5 nucleotides 360 - 4021 are replaced by either the CMV promoter and tripartite leader cDNA (TPL) or the α -fetoprotein enhancer and promoter (AFP) driving expression of the HSV-1 TK or CAT gene as
15 indicated. The resulting recombinant adenoviruses are designated ACNTK, AANTK, and AANCAT respectively.

Figure 13 shows promoter specificity of CAT expression in the recombinant adenoviral vectors. Two (2) $\times 10^6$ of the designated cell lines were infected at MOIs =
20 30 or 100 of the recombinant adenovirus AANCAT as indicated or left uninfected (UN). Hep G2 and Hep 3B cells express α -fetoprotein whereas the other cell lines do not. After three days, the cells were harvested, extract volumes were adjusted for equal total protein concentrations, and CAT
25 activity was measured as described in Methods section, below. An equal number of uninfected cells served as individual controls for background CAT activity, while 14 C labelled chloramphenicol (14 C-only) and extract from a stable cell line (B21) expressing CAT activity served as
30 negative and positive controls respectively. Percent conversion of acetyl CoA is indicated, demonstrating that CAT expression is limited to those cells expressing α -fetoprotein.

Figure 14 shows the effects of TK/GCV treatment on hepatocellular carcinoma cell lines and the effects of promoter specificity. Hep-G2 (AFP positive) and HLF (AFP negative) cell lines were infected overnight with ACNTK [- Δ -] AANTK [- Δ -], or control ACN [- \square -] virus at an infection multiplicity of 30 and subsequently treated with a single dose of ganciclovir at the indicated concentrations. Cell proliferation was assessed by adding ^3H -thymidine to the cells approximately 18 hours prior to harvest. ^3H -thymidine incorporation into cellular nucleic acid was measured 72 hours after infection (Top Count, Packard and expressed as a percent (mean \pm S.D.) of untreated control. The results show a non-selective dose dependent inhibition of proliferation with the CMV driven construct, while AFP driven TK selectively inhibits Hep-G2.

Figure 15 shows cytotoxicity of ACNTK plus ganciclovir in HCC. HLF cells were infected at an MOI of 30 with either ACNTK [- \bullet -] or the control virus ACN [- \square -] and treated with ganciclovir at the indicated doses. Seventy-two (72) hours after ganciclovir treatment, the amount of lactate dehydrogenase (LDH) released into the cell supernatant were measured colorimetrically and plotted (mean \pm -SEM) versus ganciclovir concentration for the two virus treated groups.

Figures 16A and 16B show the effect of ACNTK plus ganciclovir on established hepatocellular carcinoma (HCC) tumors in nude mice. One (1×10^7) Hep 3B cells were injected subcutaneously into the flank of female nude mice and allowed to grow for 27 days. Mice then received intratumoral and peritumoral injections of either the ACNTK [- \bullet -] or control ACN [- \square -] virus (1×10^9 iu in 100 μl volume) every other day for a total of three doses (indicated by arrows). Injections of ganciclovir (100 mg/kg ip) began 24 hours after the initial virus dose and continued for a total of 10 days. In Figure 6A, tumor

sizes are plotted for each virus versus days post infection (mean \pm SEM). In Figure 6B, body weight for each virus-treated animal group is plotted as the mean \pm SEM versus days post infection.

5 DETAILED DESCRIPTION OF THE INVENTION

To reduce the frequency of contamination with wild-type adenovirus, it is desirable to improve either the virus or the cell line to reduce the probability of recombination. For example, an adenovirus from a group
10 with low homology to the group C viruses could be used to engineer recombinant viruses with little propensity for recombination with the Ad5 sequences in 293 cells. However, an alternative, easier means of reducing the recombination between viral and cellular sequences is to
15 increase the size of the deletion in the recombinant virus and thereby reduce the extent of shared sequence between it and the Ad5 genes in the 293 cells.

Deletions which extend past 3.5 kb from the 5' end of the adenoviral genome affect the gene for adenoviral
20 protein IX and have not been considered desirable in adenoviral vectors (see below).

The protein IX gene of the adenoviruses encodes a minor component of the outer adenoviral capsid which stabilizes the group-of-nine hexons which compose the
25 majority of the viral capsid (Stewart (1993)). Based upon study of adenovirus deletion mutants, protein IX initially was thought to be a non-essential component of the adenovirus, although its absence was associated with greater heat lability than observed with wild-type virus
30 (Colby and Shenk (1981)). More recently it was discovered that protein IX is essential for packaging full length viral DNA into capsids and that in the absence of protein IX, only genomes at least 1 kb smaller than wild-type could

be propagated as recombinant viruses (Ghosh-Choudhury et al. (1987)). Given this packaging limitation, protein IX deletions deliberately have not been considered in the design of adenoviral vectors.

5 In this application, reference is made to standard textbooks of molecular biology that contain definitions, methods and means for carrying out basic techniques, encompassed by the present invention. See for example, Sambrook et al. (1989) and the various references
10 cited therein. This reference and the cited publications are expressly incorporated by reference into this disclosure.

Contrary to what has been known in the art, this invention claims the use of recombinant adenoviruses
15 bearing deletions of the protein IX gene as a means of reducing the risk of wild-type adenovirus contamination in virus preparations for use in diagnostic and therapeutic applications such as gene therapy. As used herein, the term "recombinant" is intended to mean a progeny formed as
20 the result of genetic engineering. These deletions can remove an additional 500 to 700 base pairs of DNA sequence that is present in conventional E1 deleted viruses (smaller, less desirable, deletions of portions of the pIX gene are possible and are included within the scope of this
25 invention) and is available for recombination with the Ad5 sequences integrated in 293 cells. Recombinant adenoviruses based on any group C virus, serotype 1, 2, 5 and 6, are included in this invention. Also encompassed by this invention is a hybrid Ad2/Ad5 based recombinant virus
30 expressing the human p53 cDNA from the adenovirus type 2 major late promoter. This construct was assembled as shown in Figure 1. The resultant virus bears a 5' deletion of adenoviral sequences extending from about nucleotide 357 to 4020 and eliminates the E1a and E1b genes as well as the
35 entire protein IX coding sequence, leaving the

polyadenylation site shared by the E1b and protein IX genes intact for use in terminating transcription of any desired gene. A separate embodiment is shown in Figure 4. Alternatively, the deletion can be extended an additional
5 30 to 40 base pairs without affecting the adjacent gene for protein IVa2, although in that case an exogenous polyadenylation signal is provided to terminate transcription of genes inserted into the recombinant virus. The initial virus constructed with this deletion is easily
10 propagated in 293 cells with no evidence of wild-type viral contamination and directs robust p53 expression from the transcriptional unit inserted at the site of the deletion.

The insert capacity of recombinant viruses bearing the protein IX deletion described above is
15 approximately 2.6 kb. This is sufficient for many genes including the p53 cDNA. Insert capacity can be increased by introducing other deletions into the adenoviral backbone, for example, deletions within early regions 3 or 4 (for review see: Graham and Prevec (1991)). For
20 example, the use of an adenoviral backbone containing a 1.9 kb deletion of non-essential sequence within early region 3. With this additional deletion, the insert capacity of the vector is increased to approximately 4.5 kb, large enough for many larger cDNAs, including that of the
25 retinoblastoma tumor suppressor gene.

A recombinant adenovirus expression vector characterized by the partial or total deletion of the adenoviral protein IX DNA and having a gene encoding a foreign protein, or a functional fragment or mutant thereof
30 is provided by this invention. These vectors are useful for the safe recombinant production of diagnostic and therapeutic polypeptides and proteins, and more importantly, for the introduction of genes in gene therapy. Thus, for example, the adenoviral vector of this invention
35 can contain a foreign gene for the expression of a protein

effective in regulating the cell cycle, such as p53, Rb, or mitotin, or in inducing cell death, such as the conditional suicide gene thymidine kinase. (The latter must be used in conjunction with a thymidine kinase metabolite in order to be effective). Any expression cassette can be used in the vectors of this invention. An "expression cassette" means a DNA molecule having a transcription promoter/enhancer such as the CMV promoter enhancer, etc., a foreign gene, and in some embodiments defined below, a polyadenylation signal. As used herein, the term "foreign gene" is intended to mean a DNA molecule not present in the exact orientation and position as the counterpart DNA molecule found in wild-type adenovirus. The foreign gene is a DNA molecule up to 4.5 kilobases. "Expression vector" means a vector that results in the expression of inserted DNA sequences when propagated in a suitable host cell, i.e., the protein or polypeptide coded for by the DNA is synthesized by the host's system. The recombinant adenovirus expression vector can contain part of the gene encoding adenovirus protein IX, provided that biologically active protein IX or fragment thereof is not produced. Example of this vector are an expression vector having the restriction enzyme map of Figures 1 or 4.

Inducible promoters also can be used in the adenoviral vector of this invention. These promoters will initiate transcription only in the presence of an additional molecule. Examples of inducible promoters include those obtainable from a β -interferon gene, a heat shock gene, a metallothionein gene or those obtainable from steroid hormone-responsive genes. Tissue specific expression has been well characterized in the field of gene expression and tissue specific and inducible promoters such as these are very well known in the art. These genes are used to regulate the expression of the foreign gene after it has been introduced into the target cell.

Also provided by this invention is a recombinant adenovirus expression vector, as described above, having less extensive deletions of the protein IX gene sequence extending from 3500 bp from the 5' viral termini to approximately 4000 bp, in one embodiment. In a separate embodiment, the recombinant adenovirus expression vector can have a further deletion of a non-essential DNA sequence in adenovirus early region 3 and/or 4 and/or deletion of the DNA sequences designated adenovirus E1a and E1b. In this embodiment, foreign gene is a DNA molecule of a size up to 4.5 kilobases.

A further embodiment has a deletion of up to forty nucleotides positioned 3' to the E1a and E1b deletion and pIX and a foreign DNA molecule encoding a polyadenylation signal inserted into the recombinant vector in a position relative to the foreign gene to regulate the expression of the foreign gene.

For the purposes of this invention, the recombinant adenovirus expression vector can be derived from wild-type group adenovirus, serotype 1, 2, 5 or 6.

In one embodiment, the recombinant adenovirus expression vector has a foreign gene coding for a functional tumor suppressor protein, or a biologically active fragment thereof. As used herein, the term "functional" as it relates to a tumor suppressor gene, refers to tumor suppressor genes that encode tumor suppressor proteins that effectively inhibit a cell from behaving as a tumor cell. Functional genes can include, for instance, wild type of normal genes and modifications of normal genes that retains its ability to encode effective tumor suppressor proteins and other anti-tumor genes such as a conditional suicide protein or a toxin.

Similarly, "non-functional" as used herein is synonymous with "inactivated." Non-functional or defective genes can be caused by a variety of events, including for example point mutations, deletions, methylation and others
5 known to those skilled in the art.

As used herein, an "active fragment" of a gene includes smaller portions of the gene that retain the ability to encode proteins having tumor suppressing activity. p56^{RB}, described more fully below, is but one
10 example of an active fragment of a functional tumor suppressor gene. Modifications of tumor suppressor genes are also contemplated within the meaning of an active fragment, such as additions, deletions or substitutions, as long as the functional activity of the unmodified gene is
15 retained.

Another example of a tumor suppressor gene is retinoblastoma (RB). The complete RB cDNA nucleotide sequences and predicted amino acid sequences of the resulting RB protein (designated p110^{RB}) are shown in Lee et
20 al. (1987) and in Figure 3. Also useful to express retinoblastoma tumor suppressor protein is a DNA molecule encoding the amino acid sequence shown in Figure 2 or having the DNA sequence shown in Figure 3. A truncated version of p110^{RB}, called p56^{RB} also is useful. For the
25 sequence of p56^{RB}, see Huang et al. (1991). Additional tumor suppressor genes can be used in the vectors of this invention. For illustration purposes only, these can be p16 protein (Kamb et al. (1994)), p21 protein, Wilm's tumor WT1 protein, mitosin, h-NUC, or colon carcinoma DCC
30 protein. Mitosin is described in X. Zhu and W-H Lee, U.S. Application Serial No. 08/141,239, filed October 22, 1993, and a subsequent continuation-in-part by the same inventors, attorney docket number P-CJ 1191, filed October 24, 1994, both of which are herein incorporated by
35 reference. Similarly, h-NUC is described by W-H Lee and P-

L Chen, U.S. Application Serial No. 08/170,586, filed December 20, 1993, herein incorporated by reference.

As is known to those of skill in the art, the term "protein" means a linear polymer of amino acids joined in a specific sequence by peptide bonds. As used herein, the term "amino acid" refers to either the D or L stereoisomer form of the amino acid, unless otherwise specifically designated. Also encompassed within the scope of this invention are equivalent proteins or equivalent peptides, e.g., having the biological activity of purified wild type tumor suppressor protein. "Equivalent proteins" and "equivalent polypeptides" refer to compounds that depart from the linear sequence of the naturally occurring proteins or polypeptides, but which have amino acid substitutions that do not change its biological activity. These equivalents can differ from the native sequences by the replacement of one or more amino acids with related amino acids, for example, similarly charged amino acids, or the substitution or modification of side chains or functional groups.

Also encompassed within the definition of a functional tumor suppressor protein is any protein whose presence reduces the tumorigenicity, malignancy or hyperproliferative phenotype of the host cell. Examples of tumor suppressor proteins within this definition include, but are not limited to p110^{RB}, p56^{RB}, mitotin, h-NUC and p53. "Tumorigenicity" is intended to mean having the ability to form tumors or capable of causing tumor formation and is synonymous with neoplastic growth. "Malignancy" is intended to describe a tumorigenic cell having the ability to metastasize and endanger the life of the host organism. "Hyperproliferative phenotype" is intended to describe a cell growing and dividing at a rate beyond the normal limitations of growth for that cell type. "Neoplastic" also is intended to include cells lacking endogenous

functional tumor suppressor protein or the inability of the cell to express endogenous nucleic acid encoding a functional tumor suppressor protein.

An example of a vector of this invention is a
5 recombinant adenovirus expression vector having a foreign gene coding for p53 protein or an active fragment thereof is provided by this invention. The coding sequence of the p53 gene is set forth below in Table I.

TABLE 1

50
V*SHR PGSR* LLGSG DTLRS GWERA FHDGD TLPWI GSQTA FRVTA MEEPQ
100
5 SDPSV EPPLS QETFS DLWKL LPENN VLSPL PSQAM DDLML SPDDI EQWFT
150
EDPGP DEAPR MPEAA PPVAP APAAP TPAAP APAPS WPLSS SVPSQ KTYQG
200
SYGFR LGFLH SGTAK SVTCT YSPAL NKMFC QLAKT CPVQL WVDST PPPGT
10 250
RVRAM AIYKQ SQHMT EVVRR CPHHE RCSDS DGLAP PQHLI RVEGN LRVEY
300
LDDRN TFRHS VVVPY EPPEV GSDCT TIHYN YMCNS SCMGG MNRRP ILTII
350
15 TLEDs SGNLL GRNSF EVRVC ACPGR DRRTE EENLR KKGEP HHELP PGSTK
400
RALPN NTSSS PQPKK KPLDG EYFTL QIRGR ERFEM FRELN EALEL KDAQA

GKEPG GSRAH SSHLK SKKGQ STSRH KKLmf KTEGP DSD*

* = Stop codon

20 Any of the expression vectors described herein
are useful as compositions for diagnosis or therapy. The
vectors can be used for screening which of many tumor
suppressor genes would be useful in gene therapy. For
example, a sample of cells suspected of being neoplastic
25 can be removed from a subject and mammal. The cells can
then be contacted, under suitable conditions and with an
effective amount of a recombinant vector of this invention
having inserted therein a foreign gene encoding one of
several functional tumor suppressor genes. Whether the
30 introduction of this gene will reverse the malignant
phenotype can be measured by colony formation in soft agar
or tumor formation in nude mice. If the malignant
phenotype is reversed, then that foreign gene is determined
to be a positive candidate for successful gene therapy for
35 the subject or mammal. When used pharmaceutically, they

can be combined with one or more pharmaceutically acceptable carriers. Pharmaceutically acceptable carriers are well known in the art and include aqueous solutions such as physiologically buffered saline or other solvents or vehicles such as glycols, glycerol, vegetable oils (eg., olive oil) or injectable organic esters. A pharmaceutically acceptable carrier can be used to administer the instant compositions to a cell *in vitro* or to a subject *in vivo*.

10 A pharmaceutically acceptable carrier can contain a physiologically acceptable compound that acts, for example, to stabilize the composition or to increase or decrease the absorption of the agent. A physiologically acceptable compound can include, for example, 15 carbohydrates, such as glucose, sucrose or dextrans, antioxidants, such as ascorbic acid or glutathione, chelating agents, low molecular weight proteins or other stabilizers or excipients. Other physiologically acceptable compounds include wetting agents, emulsifying 20 agents, dispersing agents or preservatives, which are particularly useful for preventing the growth or action of microorganisms. Various preservatives are well known and include, for example, phenol and ascorbic acid. One skilled in the art would know that the choice of a 25 pharmaceutically acceptable carrier, including a physiologically acceptable compound, depends, for example, on the route of administration of the polypeptide and on the particular physio-chemical characteristics of the specific polypeptide. For example, a physiologically 30 acceptable compound such as aluminum monostearate or gelatin is particularly useful as a delaying agent, which prolongs the rate of absorption of a pharmaceutical composition administered to a subject. Further examples of carriers, stabilizers or adjuncts can be found in Martin, 35 Remington's Pharm. Sci., 15th Ed. (Mack Publ. Co., Easton, 1975), incorporated herein by reference. The

pharmaceutical composition also can be incorporated, if desired, into liposomes, microspheres or other polymer matrices (Gregoriadis, Liposome Technology, Vol. 1 (CRC Press, Boca Raton, Florida 1984), which is incorporated
5 herein by reference). Liposomes, for example, which consist of phospholipids or other lipids, are nontoxic, physiologically acceptable and metabolizable carriers that are relatively simple to make and administer.

As used herein, "pharmaceutical composition"
10 refers to any of the compositions of matter described herein in combination with one or more of the above pharmaceutically acceptable carriers. The compositions can then be administered therapeutically or prophylactically. They can be contacted with the host cell *in vivo*, *ex vivo*,
15 or *in vitro*, in an effective amount. *In vitro* and *ex vivo* means of contacting host cells are provided below. When practiced *in vivo*, methods of administering a pharmaceutical containing the vector of this invention, are well known in the art and include but are not limited to,
20 administration orally, intra-tumorally, intravenously, intramuscularly or intraperitoneal. Administration can be effected continuously or intermittently and will vary with the subject and the condition to be treated, e.g., as is the case with other therapeutic compositions (Landmann et
25 al. (1992); Aulitzky et al. (1991); Lantz et al. (1990); Supersaxo et al. (1988); Demetri et al. (1989); and LeMaistre et al. (1991)).

Further provided by this invention is a transformed procaryotic or eucaryotic host cell, for
30 example an animal cell or mammalian cell, having inserted a recombinant adenovirus expression vector described above. Suitable procaryotic cells include but are not limited to bacterial cells such as *E. coli* cells. Methods of transforming host cells with retroviral vectors are known
35 in the art, see Sambrook et al. (1989) and include, but are

not limited to transfection, electroporation, and microinjection.

As used throughout this application, the term animal is intended to be synonymous with mammal and is to include, but not be limited to bovine, porcine, feline, simian, canine, equine, murine, rat or human. Additional host cells include but are not limited to any neoplastic or tumor cell, such as osteosarcoma, ovarian carcinoma, breast carcinoma, melanoma, hepatocarcinoma, lung cancer, brain cancer, colorectal cancer, hematopoietic cell, prostate cancer, cervical carcinoma, retinoblastoma, esophageal carcinoma, bladder cancer, neuroblastoma, or renal cancer.

Additionally, any eucaryotic cell line capable of expressing Ela and Elb or Ela, Elb and pIX is a suitable host for this vector. In one embodiment, a suitable eucaryotic host cell is the 293 cell line available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A. 20231.

Any of the transformed host cells described herein are useful as compositions for diagnosis or therapy. When used pharmaceutically, they can be combined with various pharmaceutically acceptable carriers. Suitable pharmaceutically acceptable carriers are well known to those of skill in the art and, for example, are described above. The compositions can then be administered therapeutically or prophylactically, in effective amounts, described in more detail below.

A method of transforming a host cell also is provided by this invention. This method provides contacting a host cell, i.e., a procaryotic or eucaryotic host cell, with any of the expression vectors described herein and under suitable conditions. Host cells transformed by this method also are claimed within the

scope of this invention. The contacting can be effected *in vitro*, *in vivo*, or *ex vivo*, using methods well known in the art (Sambrook et al. (1989)) and using effective amounts of the expression vectors. Also provided in this invention is

5 a method of producing a recombinant protein or polypeptide by growing the transformed host cell under suitable conditions favoring the transcription and translation of the inserted foreign gene. Methods of recombinant expression in a variety of host cells, such as mammalian,

10 yeast, insect or bacterial cells, are widely known, including those described in Sambrook et al., supra. The translated foreign gene can then be isolated by convention means, such as column purification or purification using an anti-protein antibody. The isolated protein or polypeptide

15 also is intended within the scope of this invention. As used herein, purified or isolated mean substantially free of native proteins or nucleic acids normally associated with the protein or polypeptide in the native or host cell environment.

20 Also provided by this invention are non-human animals having inserted therein the expression vectors or transformed host cells of this invention. These "transgenic" animals are made using methods well known to those of skill in the art, for example as described in U.S.

25 Patent No. 5,175,384 or by conventional *ex vivo* therapy techniques, as described in Culver et al. (1991).

As shown in detail below, the recombinant adenoviruses expressing a tumor suppressor wild-type p53, as described above, can efficiently inhibit DNA synthesis

30 and suppress the growth of a broad range of human tumor cell types, including clinical targets. Furthermore, recombinant adenoviruses can express tumor suppression genes such as p53 in an *in vivo* established tumor without relying on direct injection into the tumor or prior *ex vivo*

35 treatment of the cancer cells. The p53 expressed is

functional and effectively suppresses tumor growth *in vivo* and significantly increases survival time in a nude mouse model of human lung cancer.

Thus, the vectors of this invention are particularly suited for gene therapy. Accordingly, methods of gene therapy utilizing these vectors are within the scope of this invention. The vector is purified and then an effective amount is administered *in vivo* or *ex vivo* into the subject. Methods of gene therapy are well known in the art, see, for example, Larrick, J.W. and Burck, K.L. (1991) and Kreigler, M. (1990). "Subject" means any animal, mammal, rat, murine, bovine, porcine, equine, canine, feline or human patient. When the foreign gene codes for a tumor suppressor gene or other anti-tumor protein, the vector is useful to treat or reduce hyperproliferative cells in a subject, to inhibit tumor proliferation in a subject or to ameliorate a particular related pathology. Pathologic hyperproliferative cells are characteristic of the following disease states, thyroid hyperplasia - Grave's Disease, psoriasis, benign prostatic hypertrophy, Li-Fraumeni syndrome including breast cancer, sarcomas and other neoplasms, bladder cancer, colon cancer, lung cancer, various leukemias and lymphomas. Examples of non-pathologic hyperproliferative cells are found, for instance, in mammary ductal epithelial cells during development of lactation and also in cells associated with wound repair. Pathologic hyperproliferative cells characteristically exhibit loss of contact inhibition and a decline in their ability to selectively adhere which implies a change in the surface properties of the cell and a further breakdown in intercellular communication. These changes include stimulation to divide and the ability to secrete proteolytic enzymes.

Moreover, the present invention relates to a method for depleting a suitable sample of pathologic

mammalian hyperproliferative cells contaminating hematopoietic precursors during bone marrow reconstitution via the introduction of a wild type tumor suppressor gene into the cell preparation using the vector of this invention (whether derived from autologous peripheral blood or bone marrow). As used herein, a "suitable sample" is defined as a heterogeneous cell preparation obtained from a patient, e.g., a mixed population of cells containing both phenotypically normal and pathogenic cells.

10 "Administer" includes, but is not limited to introducing into the cell or subject intravenously, by direct injection into the tumor, by intra-tumoral injection, by intraperitoneal administration, by aerosol administration to the lung or topically. Such administration can be

15 combined with a pharmaceutically-accepted carrier, described above.

The term "reduced tumorigenicity" is intended to mean tumor cells that have been converted into less tumorigenic or non-tumorigenic cells. Cells with reduced

20 tumorigenicity either form no tumors *in vivo* or have an extended lag time of weeks to months before the appearance of *in vivo* tumor growth and/or slower growing three dimensional tumor mass compared to tumors having fully inactivated or non-functional tumor suppressor gene.

25 As used herein, the term "effective amount" is intended to mean the amount of vector or anti-cancer protein which achieves a positive outcome on controlling cell proliferation. For example, one dose contains from about 10^8 to about 10^{13} infectious units. A typical course

30 of treatment would be one such dose a day over a period of five days. An effective amount will vary on the pathology or condition to be treated, by the patient and his status, and other factors well known to those of skill in the art. Effective amounts are easily determined by those of skill

35 in the art.

Also within the scope of this invention is a method of ameliorating a pathology characterized by hyperproliferative cells or genetic defect in a subject by administering to the subject an effective amount of a
5 vector described above containing a foreign gene encoding a gene product having the ability to ameliorate the pathology, under suitable conditions. As used herein, the term "genetic defect" means any disease or abnormality that results from inherited factors, such as sickle cell anemia
10 or Tay-Sachs disease.

This invention also provides a method for reducing the proliferation of tumor cells in a subject by introducing into the tumor mass an effective amount of an adenoviral expression vector containing an anti-tumor gene
15 other than a tumor suppressor gene. The anti-tumor gene can encode, for example, thymidine kinase (TK). The subject is then administered an effective amount of a therapeutic agent, which in the presence of the anti-tumor gene is toxic to the cell. In the specific case of
20 thymidine kinase, the therapeutic agent is a thymidine kinase metabolite such as ganciclovir (GCV), 6-methoxypurine arabinonucleoside (araM), or a functional equivalent thereof. Both the thymidine kinase gene and the thymidine kinase metabolite must be used concurrently to be
25 toxic to the host cell. However, in its presence, GCV is phosphorylated and becomes a potent inhibitor of DNA synthesis whereas araM gets converted to the cytotoxic anabolite araATP. Other anti-tumor genes can be used as well in combination with the corresponding therapeutic
30 agent to reduce the proliferation of tumor cells. Such other gene and therapeutic agent combinations are known by one skilled in the art. Another example would be the vector of this invention expressing the enzyme cytosine deaminase. Such vector would be used in conjunction with
35 administration of the drug 5-fluorouracil (Austin and Huber, 1993), or the recently described E. Coli Deo Δ gene

in combination with 6-methyl-purine-2'-deosribonucleoside (Sorscher et al 1994).

As with the use of the tumor suppressor genes described previously, the use of other anti-tumor genes, either alone or in combination with the appropriate therapeutic agent provides a treatment for the uncontrolled cell growth or proliferation characteristic of tumors and malignancies. Thus, this invention provides a therapy to stop the uncontrolled cellular growth in the patient thereby alleviating the symptoms of the disease or cachexia present in the patient. The effect of this treatment includes, but is not limited to, prolonged survival time of the patient, reduction in tumor mass or burden, apoptosis of tumor cells or the reduction of the number of circulating tumor cells. Means of quantifying the beneficial effects of this therapy are well known to those of skill in the art.

The invention provides a recombinant adenovirus expression vector characterized by the partial or total deletion of the adenoviral protein IX DNA and having a foreign gene encoding a foreign protein, wherein the foreign protein is a suicide gene or functional equivalent thereof. The anti-cancer gene TK, described above, is an example of a suicide gene because when expressed, the gene product is, or can be made to be lethal to the cell. For TK, lethality is induced in the presence of GCV. The TK gene is derived from herpes simplex virus by methods well known to those of skill in the art. The plasmid pMLBKTK in E. coli HB101 (from ATCC #39369) is a source of the herpes simplex virus (HSV-1) thymidine kinase (TK) gene for use in this invention. However, many other sources exist as well.

The TK gene can be introduced into the tumor mass by combining the adenoviral expression vector with a

suitable pharmaceutically acceptable carrier. Introduction can be accomplished by, for example, direct injection of the recombinant adenovirus into the tumor mass. For the specific case of a cancer such as hepatocellular carcinoma (HCC), direct injection into the hepatic artery can be used for delivery because most HCCs derive their circulation from this artery. To control proliferation of the tumor, cell death is induced by treating the patients with a TK metabolite such as ganciclovir to achieve reduction of tumor mass. The TK metabolite can be administered, for example, systemically, by local inoculation into the tumor or in the specific case of HCC, by injection into the hepatic artery. The TK metabolite is preferably administered at least once daily but can be increased or decreased according to the need. The TK metabolite can be administered simultaneous or subsequent to the administration of the TK containing vector. Those skilled in the art know or can determine the dose and duration which is therapeutically effective.

A method of tumor-specific delivery of a tumor suppressor gene is accomplished by contacting target tissue in an animal with an effective amount of the recombinant adenoviral expression vector of this invention. The gene is intended to code for an anti-tumor agent, such as a functional tumor suppressor gene or suicide gene. "Contacting" is intended to encompass any delivery method for the efficient transfer of the vector, such as intra-tumoral injection.

The use of the adenoviral vector of this invention to prepare medicaments for the treatment of a disease or for therapy is further provided by this invention.

The following examples are intended to illustrate, not limit the scope of this invention.

EXPERIMENT NO. I

Plasmid pAd/MLP/p53/E1b- was used as the starting material for these manipulations. This plasmid is based on the pBR322 derivative pML2 (pBR322 deleted for base pairs 1140 to 2490) and contains adenovirus type 5 sequences extending from base pair 1 to base pair 5788 except that it is deleted for adenovirus type 5 base pairs 357 to 3327. At the site of the Ad5 357/3327 deletion a transcriptional unit is inserted which is comprised of the adenovirus type 2 major late promoter, the adenovirus type 2 tripartite leader cDNA and the human p53 cDNA. It is a typical E1 replacement vector deleted for the Ad5 E1a and E1b genes but containing the Ad5 protein IX gene (for review of Adenovirus vectors see: Graham and Prevec (1992)). Ad2 DNA was obtained from Gibco BRL. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs. *E. coli* DH5 α competent cells were purchased from Gibco BRL and 293 cells were obtained from the American Type Culture Collection (ATCC). Prep-A-Gene DNA purification resin was obtained from BioRad. LB broth bacterial growth medium was obtained from Difco. Qiagen DNA purification columns were obtained from Qiagen, Inc. Ad5 dl327 was obtained from R.J. Schneider, NYU. The MBS DNA transfection kit was purchased from Stratagene.

One (1) μ g pAd/MLP/p53/E1b- was digested with 20 units each of restriction enzymes Ecl 136II and NgoMI according to the manufacturer's recommendations. Five (5) μ g Ad2 DNA was digested with 20 units each of restriction endonucleases DraI and NgoMI according to the manufacturer's recommendations. The restriction digestions were loaded into separate lanes of a 0.8% agarose gel and electrophoresed at 100 volts for 2 hours. The 4268 bp restriction fragment from the Pad/MLP/p53/E1b- sample and the 6437 bp fragment from the Ad2 sample were isolated from the gel using Prep-A-Gene DNA extraction resin according to

the manufacturer's specifications. The restriction fragments were mixed and treated with T4 DNA ligase in a total volume of 50 μ l at 16°C for 16 hours according to the manufacturer's recommendations. Following ligation 5 μ l of the reaction was used to transform *E. coli* DH5 α cells to ampicillin resistance following the manufacturer's procedure. Six bacterial colonies resulting from this procedure were used to inoculate separate 2 ml cultures of LB growth medium and incubated overnight at 37°C with shaking. DNA was prepared from each bacterial culture using standard procedures (Sambrook et al. (1989)). One fourth of the plasmid DNA from each isolate was digested with 20 units of restriction endonuclease XhoI to screen for the correct recombinant containing XhoI restriction fragments of 3627, 3167, 2466 and 1445 base pairs. Five of six screened isolates contained the correct plasmid. One of these was then used to inoculate a 1 liter culture of LB medium for isolation of large quantities of plasmid DNA. Following overnight incubation plasmid DNA was isolated from the 1 liter culture using Qiagen DNA purification columns according to the manufacturer's recommendations. The resulting plasmid was designated Pad/MLP/p53/PIX-. Samples of this plasmid were deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A., 12301, on October 22, 1993. The deposit was made under the provisions of the Budapest Treaty on the International Deposit of Microorganisms for the Purpose of Patent Procedure. The deposit was accorded ATCC Accession No. 75576.

To construct a recombinant adenovirus, 10 μ g Pad/MLP/p53/PIX- were treated with 40 units of restriction endonuclease EcoRI to linearize the plasmid. Adenovirus type 5 dl327 DNA (Thimmappaya (1982)) was digested with restriction endonuclease ClaI and the large fragment (approximately 33 kilobase pairs) was purified by sucrose gradient centrifugation. Ten (10) μ g of EcoRI treated

MATERIALS AND METHODS

Recombinant adenoviruses were grown and propagated in the human embryonal kidney cell line 293 (ATCC CRL 1573) maintained in DME medium containing 10% defined, supplemented calf serum (Hyclone). Saos-2 cells were maintained in Kaighn's media supplemented with 15% fetal calf serum. HeLa and Hep 3B cells were maintained in DME medium supplemented with 10% fetal calf serum. All other cell lines were grown in Kaighn's media supplemented with 10% fetal calf serum. Saos-2 cells were kindly provided by Dr. Eric Stanbridge. All other cell lines were obtained from ATCC.

30 To construct the Ad5/p53 viruses, a 1.4 kb
HindIII-SmaI fragment containing the full length cDNA for

p53 (Table I) was isolated from pGEM1-p53-B-T (kindly supplied by Dr. Wen Hwa Lee) and inserted into the multiple cloning site of the expression vector pSP72 (Promega) using standard cloning procedures (Sambrook et al. (1989)). The p53 insert was recovered from this vector following digestion with XhoI-BglII and gel electrophoresis. The p53 coding sequence was then inserted into either pNL3C or pNL3CMV adenovirus gene transfer vectors (kindly provided by Dr. Robert Schneider) which contain the Ad5 5' inverted terminal repeat and viral packaging signals and the E1a enhancer upstream of either the Ad2 major late promoter (MLP) or the human cytomegalovirus immediate early gene promoter (CMV), followed by the tripartite leader CDNA and Ad 5 sequence 3325-5525 bp in a PML2 background. These new constructs replace the E1 region (bp 360-3325) of Ad5 with p53 driven by either the Ad2 MLP (A/M/53) or the human CMV promoter (A/C/53), both followed by the tripartite leader CDNA (see Figure 4). The p53 inserts use the remaining downstream E1b polyadenylation site. Additional MLP and CMV driven p53 recombinants (A/M/N/53, A/C/N/53) were generated which had a further 705 nucleotide deletion of Ad 5 sequence to remove the protein IX (PIX) coding region. As a control, a recombinant adenovirus was generated from the parental PNL3C plasmid without a p53 insert (A/M). A second control consisted of a recombinant adenovirus encoding the beta-galactosidase gene under the control of the CMV promoter (A/C/ β -gal). The plasmids were linearized with either Nru I or Eco RI and co-transfected with the large fragment of a Cla I digested Ad 5 d1309 or d1327 mutants (Jones and Shenk (1979)) using a Ca/PO₄ transfection kit (Stratagene). Viral plaques were isolated and recombinants identified by both restriction digest analysis and PCR using recombinant specific primers against the tripartite leader CDNA sequence with downstream p53 CDNA sequence. Recombinant virus was further purified by limiting dilution, and virus particles were purified and

titered by standard methods (Graham and van der Erb (1973); Graham and Prevec (1991)).

p53 Protein Detection

Saos-2 or Hep 3B cells (5×10^5) were infected
5 with the indicated recombinant adenoviruses for a period of
24 hours at increasing multiplicities of infection (MOI) of
plaque forming units of virus/cell. Cells were then washed
once with PBS and harvested in lysis buffer (50mM Tris-HCl
Ph 7.5, 250 mM NaCl, 0.1% NP40, 50mM NaF, 5mM EDTA, 10ug/ml
10 aprotinin, 10 ug/ml leupeptin, and 1mM PMSF). Cellular
proteins (approximately 30 μ g) were separated by 10% SDS-
PAGE and transferred to nitrocellulose. Membranes were
incubated with α -p53 antibody PAb 1801 (Novocastro)
followed by sheep anti-mouse IgG conjugated with
15 horseradish peroxidase. p53 protein was visualized by
chemiluminescence (ECL kit, Amersham) on Kodak XAR-5 film.

Measurement of DNA Synthesis Rate

Cells (5×10^3 /well) were plated in 96-well titer
plates (Costar) and allowed to attach overnight (37°C, 7%
20 CO₂). Cells were then infected for 24 hours with purified
recombinant virus particles at MOIs ranging from 0.3 to 100
as indicated. Media were changed 24 hours after infection,
and incubation was continued for a total of 72 hours. ³H-
thymidine (Amersham, 1 μ Ci/well) was added 18 hours prior to
25 harvest. Cells were harvested on glass fiber filters and
levels of incorporated radioactivity were measured in a
beta scintillation counter. ³H-thymidine incorporation was
expressed as the mean % (+/- SD) of media control and
plotted versus the MOI.

30 Tumorigenicity in Nude Mice

Approximately 2.4×10^8 Saos-2 cells, plated in T225 flasks, were treated with suspension buffer (1% sucrose in PBS) containing either A/M/N/53 or A/M purified virus at an MOI of 3 or 30. Following an overnight infection, cells were injected subcutaneously into the left and right flanks of BALB/c athymic nude mice (4 mice per group). One flank was injected with the A/M/N/53 treated cells, while the contralateral flank was injected with the control A/M treated cells, each mouse serving as its own control. Animals receiving bilateral injection of buffer treated cells served as additional controls. Tumor dimensions (length, width and height) and body weights were then measured twice per week over an 8 week period. Tumor volumes were estimated for each animal assuming a spherical geometry with radius equal to one-half the average of the measured tumor dimensions.

Intra-tumoral RNA Analysis

BALB/c athymic nude mice (approximately 5 weeks of age) were injected subcutaneously with 1×10^7 H69 small cell lung carcinoma (SCLC) cells in their right flanks. Tumors were allowed to progress for 32 days until they were approximately 25-50 mm³. Mice received peritumoral injections of either A/C/53 or A/C/β-gal recombinant adenovirus (2×10^9 plaque forming units (pfu)) into the subcutaneous space beneath the tumor mass. Tumors were excised from the animals 2 and 7 days post adenovirus treatment and rinsed with PBS. Tumor samples were homogenized, and total RNA was isolated using a TriReagent kit (Molecular Research Center, Inc.). PolyA RNA was isolated using the PolyAtract mRNA Isolation System (Promega), and approximately 10 ng of sample was used for RT-PCR determination of recombinant p53 mRNA expression (Wang et al. (1989)). Primers were designed to amplify sequence between the adenovirus tripartite leader CDNA and

the downstream p53 CDNA, ensuring that only recombinant, and not endogenous p53 would be amplified.

p53 Gene Therapy of Established Tumors in Nude Mice

Approximately 1×10^7 H69 (SCLC) tumor cells in 200 μ l volumes were injected subcutaneously into female BALB/c athymic nude mice. Tumors were allowed to develop for 2 weeks, at which point animals were randomized by tumor size (N=5/group). Peritumoral injections of either A/M/N/53 or the control A/M adenovirus (2×10^9 pfu/injection) or buffer alone (1% sucrose in PBS) were administered twice per week for a total of 8 doses/group. Tumor dimensions and body weights were measured twice per week for 7 weeks, and tumor volume was estimated as described previously. Animals were then followed to observe the effect of treatment on mouse survival.

RESULTS

Construction of Recombinant p53-Adenovirus

p53 adenoviruses were constructed by replacing a portion of the E1a and E1b region of adenovirus Type 5 with p53 CDNA under the control of either the Ad2 MLP (A/M/53) or CMV (A/C/53) promoter (schematized in Figure 4). This E1 substitution severely impairs the ability of the recombinant adenoviruses to replicate, restricting their propagation to 293 cells which supply Ad 5 E1 gene products in trans (Graham et al. (1977)). After identification of p53 recombinant adenovirus by both restriction digest and PCR analysis, the entire p53 CDNA sequence from one of the recombinant adenoviruses (A/M/53) was sequenced to verify that it was free of mutations. Following this, purified preparations of the p53 recombinants were used to infect HeLa cells to assay for the presence of phenotypically wild type adenovirus. HeLa cells, which are non-permissive for

replication of E1-deleted adenovirus, were infected with $1-4 \times 10^9$ infectious units of recombinant adenovirus, cultured for 3 weeks, and observed for the appearance of cytopathic effect (CPE). Using this assay, recombinant adenovirus replication or wild type contamination was not detected, readily evident by the CPE observed in control cells infected with wild type adenovirus at a level of sensitivity of approximately 1 in 10^9 .

p53 Protein Expression from Recombinant Adenovirus

To determine if p53 recombinant adenoviruses expressed p53 protein, tumor cell lines which do not express endogenous p53 protein were infected. The human tumor cell lines Saos-2 (osteosarcoma) and Hep 3B (hepatocellular carcinoma) were infected for 24 hours with the p53 recombinant adenoviruses A/M/53 or A/C/53 at MOIs ranging 0.1 to 200 pfu/cell. Western analysis of lysates prepared from infected cells demonstrated a dose-dependent p53 protein expression in both cell types (Figure 5). Both cell lines expressed higher levels of p53 protein following infection with A/C/53 than with A/M/53 (Figure 3). No p53 protein was detected in non-infected cells. Levels of endogenous wild-type p53 are normally quite low, and nearly undetectable by Western analysis of cell extracts (Bartek et al. (1991)). It is clear however that wild-type p53 protein levels are easily detectable after infection with either A/M/53 or A/C/53 at the lower MOIs (Figure 5), suggesting that even low doses of p53 recombinant adenoviruses can produce potentially efficacious levels of p53.

p53 Dependent Morphology Changes

The reintroduction of wild-type p53 into the p53-negative osteosarcoma cell line, Saos-2, results in a characteristic enlargement and flattening of these normally

spindle-shaped cells (Chen et al. (1990)). Subconfluent Saos-2 cells (1×10^5 cells/10cm plate) were infected at an MOI of 50 with either the A/C/53 or control A/M virus, and incubated at 37°C for 72 hours until uninfected control plates were confluent. At this point, the expected morphological change was evident in the A/C/53 treated plate (Figure 6, panel C) but not in uninfected (Figure 6, panel A) or control virus-infected plates (Figure 6, panel B). This effect was not a function of cell density because a control plate initially seeded at lower density retained normal morphology at 72 hours when its confluence approximated that of the A/C/53 treated plate. Previous results had demonstrated a high level of p53 protein expression at an MOI of 50 in Saos-2 cells (Figure 5A), and these results provided evidence that the p53 protein expressed by these recombinant adenoviruses was biologically active.

p53 Inhibition of Cellular DNA Synthesis

To further test the activity of the p53 recombinant adenoviruses, their ability to inhibit proliferation of human tumor cells was assayed as measured by the uptake of ^3H -thymidine. It has previously been shown that introduction of wild-type p53 into cells which do not express endogenous wild-type p53 can arrest the cells at the G₁/S transition, leading to inhibition of uptake of labeled thymidine into newly synthesized DNA (Baker et al. (1990); Mercer et al. (1990); Diller et al. (1990)). A variety of p53-deficient tumor cell lines were infected with either A/M/N/53, A/C/N/53 or a non-p53 expressing control recombinant adenovirus (A/M). A strong, dose-dependent inhibition of DNA synthesis by both the A/M/N/53 and A/C/N/53 recombinants in 7 out of the 9 different tumor cell lines tested (Figure 7) was observed. Both constructs were able to inhibit DNA synthesis in these human tumor cells, regardless of whether they expressed mutant p53 or

failed to express p53 protein. It also was found that in this assay, the A/C/N/53 construct was consistently more potent than the A/M/N/53. In saos-2 (osteosarcoma) and MDA-MB468 (breast cancer) cells, nearly 100% inhibition of

5 DNA synthesis was achieved with the A/C/N/53 construct at an MOI as low as 10. At doses where inhibition by the control adenovirus in only 10-30%, a 50-100% reduction in DNA synthesis using either p53 recombinant adenovirus was observed. In contrast, no significant p53-specific effect

10 was observed with either construct as compared to control virus in HEP G2 cells (hepatocarcinoma cell line expressing endogenous wild-type p53, Bressac et al. (1990)), nor in the K562 (p53 null) leukemic cell line.

Tumorigenicity in Nude Mice

In a more stringent test of function for the p53 recombinant adenoviruses, tumor cells were infected *ex vivo* and then injected the cells into nude mice to assess the ability of the recombinants to suppress tumor growth *in vivo*. Saos-2 cells infected with A/M/N/53 or control A/M virus at a MOI of 3 or 30, were injected into opposite flanks of nude mice. Tumor sizes were then measured twice a week over an 8 week period. At the MOI of 30, no tumor growth was observed in the p53-treated flanks in any of the animals, while the control treated tumors continued to grow (Figure 8). The progressive enlargement of the control virus treated tumors were similar to that observed in the buffer treated control animals. A clear difference in tumor growth between the control adenovirus and the p53 recombinant at the MOI of 3, although tumors from 2 out of the 4 p53-treated mice did start to show some growth after approximately 6 weeks. Thus, the A/M/N/53 recombinant adenovirus is able to mediate p53-specific tumor suppression in an *in vivo* environment.

In Vivo Expression of Ad/p53

Although *ex vivo* treatment of cancer cells and subsequent injection into animals provided a critical test of tumor suppression, a more clinically relevant experiment is to determine if injected p53 recombinant adenovirus could infect and express p53 in established tumors *in vivo*. To address this, H69 (SCLC, p53^{mut}) cells were injected subcutaneously into nude mice, and tumors were allowed to develop for 32 days. At this time, a single injection of 2×10^9 pfu of either A/C/53 or A/C/ β -gal adenovirus was injected into the peritumoral space surrounding the tumor. Tumors were then excised at either Day 2 or Day 7 following the adenovirus injection, and polyA RNA was isolated from each tumor. RT-PCR, using recombinant-p53 specific

primers, was then used to detect p53 mRNA in the p53 treated tumors (Figure 9, lanes 1,2,4,5). No p53 signal was evident from the tumors excised from the β -gal treated animals (Figure 9, lanes 3 and 6). Amplification with
5 actin primers served as a control for the RT-PCR reaction (Figure 9, lanes 7-9), while a plasmid containing the recombinant-p53 sequence served as a positive control for the recombinant-p53 specific band (Figure 9, lane 10). This experiment demonstrates that a p53 recombinant
10 adenovirus can specifically direct expression of p53 mRNA within established tumors following a single injection into the peritumoral space. It also shows *in vivo* viral persistence for at least one week following infection with a p53 recombinant adenovirus.

15 *In Vivo* Efficacy

To address the feasibility of gene therapy of established tumors, a tumor-bearing nude mouse model was used. H69 cells were injected into the subcutaneous space on the right flank of mice, and tumors were allowed to grow
20 for 2 weeks. Mice then received peritumoral injections of buffer or recombinant virus twice weekly for a total of 8 doses. In the mice treated with buffer or control A/M virus, tumors continued to grow rapidly throughout the treatment, whereas those treated with the A/M/N/53 virus
25 grew at a greatly reduced rate (Figure 10A). After cessation of injections, the control treated tumors continued to grow while the p53 treated tumors showed little or no growth for at least one week in the absence of any additional supply of exogenous p53 (Figure 10A).
30 Although control animals treated with buffer alone had accelerated tumor growth as compared to either virus treated group, no significant difference in body weight was found between the three groups during the treatment period. Tumor ulceration in some animals limited the relevance of
35 tumor size measurements after day 42. However, continued

monitoring of the animals to determine survival time demonstrated a survival advantage for the p53-treated animals (Figure 10B). The last of the control adenovirus treated animals died on day 83, while buffer alone treated
5 controls had all expired by day 56. In contrast, all 5 animals treated with the A/M/N/53 continue to survive (day 130 after cell inoculation) (Figure 10B). Together, this data establish a p53-specific effect on both tumor growth and survival time in animals with established p53-deficient
10 tumors.

Adenovirus Vectors Expressing p53

Recombinant human adenovirus vectors which are capable of expressing high levels of wild-type p53 protein in a dose dependent manner were constructed. Each vector
15 contains deletions in the E1a and E1b regions which render the virus replication deficient (Challberg and Kelly (1979); Horowitz, (1991)). Of further significance is that these deletions include those sequences encoding the E1b 19 and 55 kd protein. The 19 kd protein is reported to be
20 involved in inhibiting apoptosis (White et al. (1992); Rao et al. (1992)), whereas the 55 kd protein is able to bind wild-type p53 protein (Sarnow et al. (1982); Heuvel et al. (1990)). By deleting these adenoviral sequences, potential inhibitors of p53 function were removed through direct
25 binding to p53 or potential inhibition of p53 mediated apoptosis. Additional constructs were made which have had the remaining 3' E1b sequence, including all protein IX coding sequence, deleted as well. Although this has been reported to reduce the packaging size capacity of
30 adenovirus to approximately 3 kb less than wild-type virus (Ghosh-Choudhury et al. (1987)), these constructs are also deleted in the E3 region so that the A/M/N/53 and A/C/N/53 constructs are well within this size range. By deleting the pIX region, adenoviral sequences homologous to those
35 contained in 293 cells are reduced to approximately 300

base pairs, decreasing the chances of regenerating replication-competent, wild-type adenovirus through recombination. Constructs lacking pIX coding sequence appear to have equal efficacy to those with pIX.

5 p53/Adenovirus Efficacy In Vitro

In concordance with a strong dose dependency for expression of p53 protein in infected cells, a dose-dependent, p53-specific inhibition of tumor cell growth was demonstrated. Cell division, was inhibited and demonstrated by the inhibition of DNA synthesis, in a wide variety of tumor cell types known to lack wild-type p53 protein expression. Bacchetti and Graham (1993) recently reported p53 specific inhibition of DNA synthesis in the ovarian carcinoma cell line SKOV-3 by a p53 recombinant adenovirus in similar experiments. In addition to ovarian carcinoma, additional human tumor cell lines were demonstrated, representative of clinically important human cancers and including lines over-expressing mutant p53 protein, can also be growth inhibited by the p53 recombinants of this invention. At MOIs where the A/C/N/53 recombinant is 90-100% effective in inhibiting DNA synthesis in these tumor types, control adenovirus mediated suppression is less than 20%.

Although Feinstein et al. (1992) reported that re-introduction of wild-type p53 could induce differentiation and increase the proportion of cells in G₁ versus S+G₂ for leukemic K562 cells, no p53 specific effect was found in this line. Horvath and Weber (1988) have reported that human peripheral blood lymphocytes are highly nonpermissive to adenovirus infection. In separate experiments, the recombinant significantly infected the non-responding K562 cells with recombinant A/C/β-gal adenovirus, while other cell lines, including the control Hep G2 line and those showing a strong p53 effect, were

readily infectable. Thus, at least part of the variability of efficacy would appear to be due to variability of infection, although other factors may be involved as well.

The results observed with the A/M/N/53 virus in Figure 8 demonstrates that complete suppression is possible in an *in vivo* environment. The resumption of tumor growth in 2 out of 4, p53 treated animals at the lower MOI most likely resulted from a small percentage of cells not initially infected with the p53 recombinant at this dose. The complete suppression seen with A/M/N/53 at the higher dose, however, shows that the ability of tumor growth to recover can be overcome.

p53/Adenovirus *In Vivo* Efficacy

Work presented here and by other groups (Chen et al. (1990); Takahashi et al. (1992)) have shown that human tumor cells lacking expression of wild-type p53 can be treated *ex vivo* with p53 and result in suppression of tumor growth when the treated cells are transferred into an animal model. Applicants present the first evidence of tumor suppressor gene therapy of an *in vivo* established tumor, resulting in both suppression of tumor growth and increased survival time. In Applicants' system, delivery to tumor cells did not rely on direct injection into the tumor mass. Rather, p53 recombinant adenovirus was injected into the peritumoral space, and p53 mRNA expression was detected within the tumor. p53 expressed by the recombinants was functional and strongly suppressed tumor growth as compared to that of control, non-p53 expressing adenovirus treated tumors. However, both p53 and control virus treated tumor groups showed tumor suppression as compared to buffer treated controls. It has been demonstrated that local expression of tumor necrosis factor (TNF), interferon- γ , interleukin (IL)-2, IL-4 or IL-7 can lead to T-cell independent transient tumor

suppression in nude mice (Hoch et al. (1992)). Exposure of monocytes to adenovirus virions are also weak inducers of IFN- α/β (reviewed in Gooding and Wold (1990)). Therefore, it is not surprising that some tumor suppression in nude mice was observed even with the control adenovirus. This virus mediated tumor suppression was not observed in the ex vivo control virus treated Saos-2 tumor cells described earlier. The p53-specific in vivo tumor suppression was dramatically demonstrated by continued monitoring of the animals in Figure 10. The survival time of the p53-treated mice was significantly increased, with 5 out of 5 animals still alive more than 130 days after cell inoculation compared to 0 out of 5 adenovirus control treated animals. The surviving animals still exhibit growing tumors which may reflect cells not initially infected with the p53 recombinant adenovirus. Higher or more frequent dosing schedules may address this. In addition, promoter shutoff (Palmer et al. (1991)) or additional mutations may have rendered these cells resistant to the p53 recombinant adenovirus treatment. For example, mutations in the recently described WAF1 gene, a gene induced by wild-type p53 which subsequently inhibits progression of the cell cycle into S phase, (El-Deiry et al. (1993); Hunter (1993)) could result in a p53-resistant tumor.

25

EXPERIMENT NO. III

This Example shows the use of suicide genes and tissue specific expression of such genes in the gene therapy methods described herein. Hepatocellular carcinoma was chosen as the target because it is one of the most common human malignancies affecting man, causing an estimated 1,250,000 deaths per year world-wide. The incidence of this cancer is very high in Southeast Asia and Africa where it is associated with Hepatitis B and C infection and exposure to aflatoxin. Surgery is currently the only treatment which offers the potential for curing

HCC, although less than 20% of patients are considered candidates for resection (Ravoet C. et al., 1993). However, tumors other than hepatocellular carcinoma are equally applicable to the methods of reducing their proliferation described herein.

CELL LINES

All cell lines but for the HLF cell line were obtained from the American Type Tissue Culture Collection (ATCC) 12301 Parklawn Drive, Rockville Maryland. ATCC accession numbers are noted in parenthesis. The human embryonal kidney cell line 293 (CRL 1573) was used to generate and propagate the recombinant adenoviruses described herein. They were maintained in DME medium containing 10% defined, supplemented calf serum (Hyclone). The hepatocellular carcinoma cell lines Hep 3B (HB 8064), Hep G2 (HB 8065), and HLF were maintained in DME/F12 medium supplemented with 10% fetal bovine serum, as were the breast carcinoma cell lines MDA-MB468 (HTB 132) and BT-549 (HTB 122). Chang liver cells (CCL 13) were grown in MEM medium supplemented with 10% fetal bovine serum. The HLF cell line was obtained from Drs. T. Morsaki and H. Kitsuki at the Kyushu University School of Medicine in Japan.

RECOMBINANT VIRUS CONSTRUCTION

Two adenoviral expression vectors designated herein as ACNTK and AANTK and devoid of protein IX function (depicted in Figure 11) are capable of directing expression of the TK suicide gene within tumor cells. A third adenovirus expression vector designated AANCAT was constructed to further demonstrate the feasibility of specifically targeting gene expression to specific cell types using adenoviral vectors. These adenoviral constructs were assembled as depicted in Figures 11 and 12 and are derivatives of those previously described for the expression of tumor suppressor genes.

For expression of the foreign gene, expression cassettes have been inserted that utilize either the human cytomegalovirus immediate early promoter/enhancer (CMV) (Boshart, M. et al., 1985) or the human alpha-fetoprotein (AFP) enhancer/promoter (Watanabe, K. et al., 1987; Nakabayashi, H. et al., 1989) to direct transcription of the TK gene or the chloramphenicol acetyltransferase gene (CAT). The CMV enhancer promoter is capable of directing robust gene expression in a wide variety of cell types while the AFP enhancer/promoter construct restricts expression to hepatocellular carcinoma cells (HCC) which express AFP in about 70-80% of the HCC patient population. In the construct utilizing the CMV promoter/enhancer, the adenovirus type 2 tripartite leader sequence also was inserted to enhance translation of the TK transcript (Berkner, K.L. and Sharp, 1985). In addition to the E1 deletion, both adenovirus vectors are additionally deleted for 1.9 kilobases (kb) of DNA in the viral E3 region. The DNA deleted in the E3 region is non-essential for virus propagation and its deletion increases the insert capacity of the recombinant virus for foreign DNA by an equivalent amount (1.9kb) (Graham and Prevec, 1991).

To demonstrate the specificity of the AFP promoter/enhancer, the virus AANCAT also was constructed where the marker gene chloramphenicol acetyltransferase (CAT) is under the control of the AFP enhancer/promoter. In the ACNTK viral construct, the Ad2 tripartite leader sequence was placed between the CMV promoter/enhancer and the TK gene. The tripartite leader has been reported to enhance translation of linked genes. The E1 substitution impairs the ability of the recombinant viruses to replicate, restricting their propagation to 293 cells which supply the Ad5 E1 gene products in trans (Graham et al., 1977).

Adenoviral Vector ACNTK: The plasmid pMLBKTK in E. coli HB101 (from ATCC #39369) was used as the source of the herpes simplex virus (HSV-1) thymidine kinase (TK) gene. TK was excised from this plasmid as a 1.7 kb gene
5 fragment by digestion with the restriction enzymes Bgl II and Pvu II and subcloned into the compatible Bam HI, EcoR V restriction sites of plasmid pSP72 (Promega) using standard cloning techniques (Sambrook et al., 1989). The TK insert was then isolated as a 1.7 kb fragment from this
10 vector by digestion with Xba I and Bgl II and cloned into Xba I, BamHI digested plasmid pACN (Wills et al. 1994). Twenty (20) μ g of this plasmid designated pACNTK were linearized with Eco RI and cotransfected into 293 cells (ATCC CRL 1573) with 5 μ g of Cla I digested ACBGL (Wills et
15 al., 1994 supra) using a CaPO₄ transfection kit (Stratagene, San Diego, California). Viral plaques were isolated and recombinants, designated ACNTK, were identified by restriction digest analysis of isolated DNA with Xho I and BsiWI. Positive recombinants were further purified by
20 limiting dilution and expanded and titered by standard methods (Graham and Prevec, 1991).

Adenoviral Vector AANTK: The α -fetoprotein promoter (AFP-P) and enhancer (AFP-E) were cloned from a human genomic DNA (Clontech) using PCR amplification with
25 primers containing restriction sites at their ends. The primers used to isolate the 210 bp AFP-E contained a Nhe I restriction site on the 5' primer and an Xba I, Xho I, Kpn I linker on the 3' primer. The 5' primer sequence was 5'-CGC GCT AGC TCT GCC CCA AAG AGC T-3. The 5' primer
30 sequence was 5'-CGC GGT ACC CTC GAG TCT AGA TAT TGC CAG TGG TGG AAG-3'. The primers used to isolate the 1763 bp AFE fragment contained a Not I restriction site on the 5' primer and a Xba I site on the 3' primer. The 5' primer sequence was 5'-CGT GCG GCC GCT GGA GGA CTT TGA GGA TGT CTG
35 TC-3'. The 3' primer sequence was 5'-CGC TCT AGA GAG ACC AGT TAG GAA GTT TTC GCA-3'. For PCR amplification, the DNA

was denatured at 97° for 7 minutes, followed by 5 cycles of amplification at 97°, 1 minute, 53°, 1 minute, 72°, 2 minutes, and a final 72°, 10 minute extension. The amplified AFE was digested with Not I and Xba I and inserted into the Not I, Xba I sites of a plasmid vector (pA/ITR/B) containing adenovirus type 5 sequences 1-350 and 3330 - 5790 separated by a polylinker containing Not I, Xho I, Xba I, Hind III, Kpn I, Bam HI, Nco I, Sma I, and Bgl II sites. The amplified AFP-E was digested with Nhe I and Kpn I and inserted into the AFP-E containing construct described above which had been digested with Xba I and Kpn I. This new construct was then further digested with Xba I and NgoMI to remove adenoviral sequences 3330 - 5780, which were subsequently replaced with an Xba I, NgoMI restriction fragment of plasmid pACN containing nucleotides 4021 - 10457 of adenovirus type 2 to construct the plasmid pAAN containing both the α -fetoprotein enhancer and promoter. This construct was then digested with Eco RI and Xba I to isolate a 2.3 kb fragment containing the Ad5 inverted terminal repeat, the AFP-E and the AFP-P which was subsequently ligated with the 8.55 kb fragment of Eco RI, Xba I digested pACNTK described above to generate pAANTK where the TK gene is driven by the α -fetoprotein enhancer and promoter in an adenovirus background. This plasmid was then linearized with Eco RI and cotransfected with the large fragment of Cla I digested ALBGL as above and recombinants, designated AANTK, were isolated and purified as described above.

Adenoviral Vector AANCAT: The chloramphenicol acetyltransferase (CAT) gene was isolated from the pCAT-Basic Vector (Promega Corporation) by an Xba I, Bam HI digest. This 1.64 kb fragment was ligated into Xba I, Bam HI digested pAAN (described above) to create pAANCAT. This plasmid was then linearized with Eco RI and cotransfected with the large fragment of Cla I digested rA/C/ β -gal to create AANCAT.

REPORTER GENE EXPRESSION: β -GALACTOSIDASE EXPRESSION:

Cells were plated at 1×10^5 cells/well in a 24-well tissue culture plate (Costar) and allowed to adhere overnight (37°C, 7% CO₂). Overnight infections of ACBGL were performed at a multiplicity of infection (MOI) of 30. After 24 hours, cells were fixed with 3.7% Formaldehyde; PBS, and stained with 1mg/ml Xgal reagent (USB). The data was scored (+, ++, +++) by estimating the percentage of positively stained cells at each MOI. [+ = 1-33%, ++ = 33-67% and +++ = >67%]

REPORTER GENE EXPRESSION: CAT EXPRESSION:

Two (2) $\times 10^6$ cells (Hep G2, Hep 3B, HLF, Chang, and MDA-MB468) were seeded onto 10 cm plates in triplicate and incubated overnight (37°C, 7% CO₂). Each plate was then infected with either AANCAT at an MOI = 30 or 100 or uninfected and allowed to incubate for 3 days. The cells were then trypsinized and washed with PBS and resuspended in 100 μ l of 0.25 M Tris pH 7.8. The samples were frozen and thawed 3 times, and the supernatant was transferred to new tubes and incubated at 60°C for 10 minutes. The samples were then spun at 4°C for 5 minutes, and the supernatants assayed for protein concentration using a Bradford assay (Bio-Rad Protein Assay Kit). Samples were adjusted to equal protein concentrations to a final volume of 75 μ l using 0.25 M Tris, 25 μ l of 4mM acetyl CoA and 1 μ l of ¹⁴C-Chloramphenicol and incubated overnight at 37°C. 500 μ l of ethyl acetate is added to each sample and mixed by vortexing, followed by centrifugation for 5 minutes at room temperature. The upper phase is then transferred to a new tube and the ethyl acetate is evaporated by centrifugation under vacuum. The reaction products are then redissolved in 25 μ l of ethyl acetate and spotted onto a thin layer chromatography (TLC) plate and the plate is then placed in a pre-equilibrated TLC chamber (95%

chloroform, 5% methanol). The solvent is then allowed to migrate to the top of the plate, the plate is then dried and exposed to X-ray film.

CELLULAR PROLIFERATION: ³H-THYMIDINE INCORPORATION

5 Cells were plated at 5×10^3 cells/well in a 96-well micro-titer plate (Costar) and allowed to incubate overnight (37C, 7% CO₂). Serially diluted ACN, ACNTK or AATK virus in DMEM; 15% FBS; 1% glutamine was used to transfect cells at an infection multiplicity of 30 for an
10 overnight duration at which point cells were dosed in triplicate with ganciclovir (Cytovene) at log intervals between 0.001 and 100 mM (micro molar). 1 μ Ci ³H-thymidine (Amersham) was added to each well 12-18 hours before
15 harvesting. At 72 hours-post infection cells were harvested onto glass-fiber filters and incorporated ³H-thymidine was counted using liquid scintillation (TopCount, Packard). Results are plotted as percent of untreated control proliferation and tabulated as the effective dose (ED₅₀±SD) for a 50 percent reduction in proliferation over
20 media controls. ED₅₀ values were estimated by fitting a logistic equation to the dose response data.

CYTOTOXICITY: LDH RELEASE

 Cells (HLF, human HCC) were plated, infected with ACN or ACNTK and treated with ganciclovir as described
25 for the proliferation assay. At 72 hours post-ganciclovir administration, cells were spun, the supernatant was removed. The levels of lactate dehydrogenase measured colometrically (Promega, Cytotox 96™). Mean (+/- S.D.) LDH release is plotted versus M.O.I.

30 IN VIVO THERAPY

Human hepatocellular carcinoma cells (Hep 3B) were injected subcutaneously into ten female (10) athymic nu/nu mice (Simonsen Laboratories, Gilroy, CA). Each animal received approximately 1×10^7 cells in the left flank. Tumors were allowed to grow for 27 days before randomizing mice by tumor size. Mice were treated with intratumoral and peritumoral injections of ACNTK or the control virus ACN (1×10^9 iu in $100 \mu\text{l}$) every other day for a total of three doses. Starting 24 hours after the initial dose of adenovirus, the mice were dosed intraperitoneally with ganciclovir (Cytovene 100 mg/kg) daily for a total of 10 days. Mice were monitored for tumor size and body weight twice weekly. Measurements on tumors were made in three dimensions using vernier calipers and volumes were calculated using the formula $\frac{4}{3} \pi r^3$, where r is one-half the average tumor dimension.

RESULTS

The recombinant adenoviruses were used to infect three HCC cell lines (HLF, Hep3B and Hep-G2). One human liver cell line (Chang) and two breast cancer cell lines were used as controls (MDAMB468 and BT549). To demonstrate the specificity of the AFP promoter/enhancer, the virus AANCAT was constructed. This virus was used to infect cells that either do (Hep 3B, HepG2) or do not (HLE, Chang, MDAMB468) express the HCC tumor marker alpha-fetoprotein (AFP). As shown in Figure 13, AANCAT directs expression of the CAT marker gene only in those HCC cells which are capable of expressing AFP (Figure 13).

The efficacy of ACNTK and AANTK for the treatment of HCC was assessed using a ^3H -thymidine incorporation assay to measure the effect of the combination of HSV-TK expression and ganciclovir treatment upon cellular proliferation. The cell lines were infected with either ACNTK or AANTK or the control virus ACN (Wills et al., 1994

supra), which does not direct expression of HSV-TK, and then treated with increasing concentrations of ganciclovir. The effect of this treatment was assessed as a function of increasing concentrations of ganciclovir, and the concentration of ganciclovir required to inhibit ³H-thymidine incorporated by 50% was determined (ED₅₀). Additionally, a relative measure of adenovirus - mediated gene transfer and expression of each cell line was determined using a control virus which directs expression of the marker gene beta-galactosidase. The data presented in Figure 14 and Table 1 below show that the ACNTK virus/ganciclovir combination treatment was capable of inhibiting cellular proliferation in all cell lines examined as compared with the control adenovirus ACN in combination with ganciclovir. In contrast, the AANTK viral vector was only effective in those HCC cell lines which have been demonstrated to express α -fetoprotein. In addition, the AANTK/GCV combination was more effective when the cells were plated at high densities.

20

TABLE 1

Cell Line	aFP	β -gal Expression	ACN	ED50 ACNTK	AANTK
MDAMB468	-	+++	>100	2	>100
BT549	-	+++	>100	<0.3	>100
HLF	-	+++	>100	0.8	>100
CHANG	-	+++	>100	22	>100
HEP-3B	-	+	80	8	8
HEP-G2 LOW	+	++	90	2	35
HEP-G2 HIGH	+	++	89	0.5	4

30

Nude mice bearing Hep3B tumors (N=5/group) were treated intratumorally and peritumorally with equivalent

doses of ACNTK or ACN control. Twenty-four hours after the first administration of recombinant adenovirus, daily treatment of ganciclovir was initiated in all mice. Tumor dimensions from each animal were measured twice weekly via calipers, and average tumor sizes are plotted in Figure 16. Average tumor size at day 58 was smaller in the ACNTK-treated animals but the difference did not reach statistical significance ($p < 0.09$, unpaired t-test). These data support a specific effect of ACNTK on tumor growth in vivo. No significant differences in average body weight were detected between the groups.

Although the invention has been described with reference to the above embodiments, it should be understood that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the claims that follow.

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What is claimed is:

1. A recombinant adenovirus expression vector comprising a partial or total deletion of a protein IX DNA and a gene encoding a foreign protein.
2. The recombinant adenovirus expression vector of claim 1, wherein the deletion of the protein IX gene sequence extends from about 3500 bp from the 5' viral termini to about 4000 bp from the 5' viral termini.
3. The recombinant adenovirus expression vector of claim 2 further comprising deletion of a non-essential DNA sequence in adenovirus early region 3 and/or early region 4.
4. The recombinant adenovirus expression vector of claim 2 further comprising deletion of a DNA sequences designated adenovirus E1a and E1b.
5. The recombinant adenovirus expression vector of claim 2 further comprising deletion of early region 3 and/or 4 and DNA sequences designated adenovirus E1a and E1b.
6. The recombinant adenovirus expression vector of claim 4 or 5 further comprising a deletion of up to forty nucleotides positioned 3' to the E1a and E1b and protein IX deletion and a foreign DNA molecule encoding a polyadenylation signal.
7. The recombinant adenovirus expression vector of claims 1 to 6, wherein the adenovirus is a Group C adenovirus selected from a serotype 1, 2, 5 or 6.

8. The recombinant adenovirus expression vector of claim 1, wherein the gene is a DNA molecule up to 2.6 kilobases.

9. The recombinant adenovirus expression vector of claim 6, wherein the gene is a DNA molecule up to 4.5 kilobases.

10. The recombinant adenovirus expression vector of claim 1, wherein the gene encodes a foreign functional protein or a biologically active fragment thereof.

11. The recombinant adenovirus expression vector of claim 10, wherein the gene encodes a foreign functional tumor suppressor protein or a biologically active fragment thereof.

12. The recombinant adenovirus expression vector of claim 1, wherein the gene encodes a suicide protein or functional equivalent thereof.

13. A transformed host cell comprising the recombinant adenovirus expression vector of claim 1 or 10.

14. The transformed host cell of claim 13, wherein the host cell is a procaryotic or eucaryotic cell.

15. A method for transforming a pathologic hyperproliferative mammalian cell comprising contacting the cell with the expression vector of claim 1.

16. A method of treating a pathology in an animal or mammal caused by the absence of a tumor suppressor gene or the presence of a pathologically mutated tumor suppressor gene comprising administering to the animal or mammal an effective amount of the vector of claim 1 containing a gene encoding a foreign functional protein

having a tumor suppressive function, under suitable conditions.

17. The method of claim 16, wherein the foreign protein is a functional tumor suppressor protein.

18. A method of gene therapy comprising administering to a subject an effective amount of the vector of claim 1.

19. A method of inhibiting the proliferation of a tumor in an animal comprising administering an effective amount of the adenoviral expression vector of claim 1 under suitable conditions to the animal.

20. The method of claim 19, wherein the gene encodes an anti-tumor agent.

21. The method of claim 20, wherein the anti-tumor agent is a tumor suppressor gene.

22. The method of claim 20, wherein the anti-tumor agent is a suicide gene or functional equivalent thereof.

23. The method of claim 21, wherein the tumor is non-small cell lung cancer, small cell lung cancer, hepatocarcinoma, melanoma, retinoblastoma, breast tumor, colorectal carcinoma, leukemia, lymphoma, brain tumor, cervical carcinoma, sarcoma, prostate tumor, bladder tumor, tumor of the reticuloendothelial tissues, Wilm's tumor, astrocytoma, glioblastoma, neuroblastoma, ovarian carcinoma, osteosarcoma, and renal cancer.

24. The method of claim 19, wherein the vector is administered by intra-tumoral injection.

25. A pharmaceutical composition comprising the recombinant adenoviral expression vector of claim 1, 10 or 12.

26. A method for reducing the proliferation of tumor cells in a subject comprising administering under suitable conditions an effective amount of an adenoviral expression vector of claim 12 and an effective amount of a
5 thymidine kinase metabolite or a functional equivalent thereof.

27. The method of claim 26, wherein the thymidine kinase metabolite is ganciclovir or 6-methoxypurine arabinonucleoside or a functional equivalent thereof.

28. The method of claim 26, wherein the adenoviral expression vector is administered by injection into the tumor mass.

29. The method of claim 26, wherein the tumor cells are hepatocellular carcinoma.

30. The method of claim 29, wherein the adenoviral expression vector is administered directly into the hepatic artery of the subject.

31. A kit for reducing the proliferation of tumor cells comprising the components of the adenoviral expression vector of claim 12, a thymidine kinase metabolite or functional equivalent thereof, pharmaceutical
5 carriers and instructions for the treatment of hepatocellular carcinoma using the kit components.

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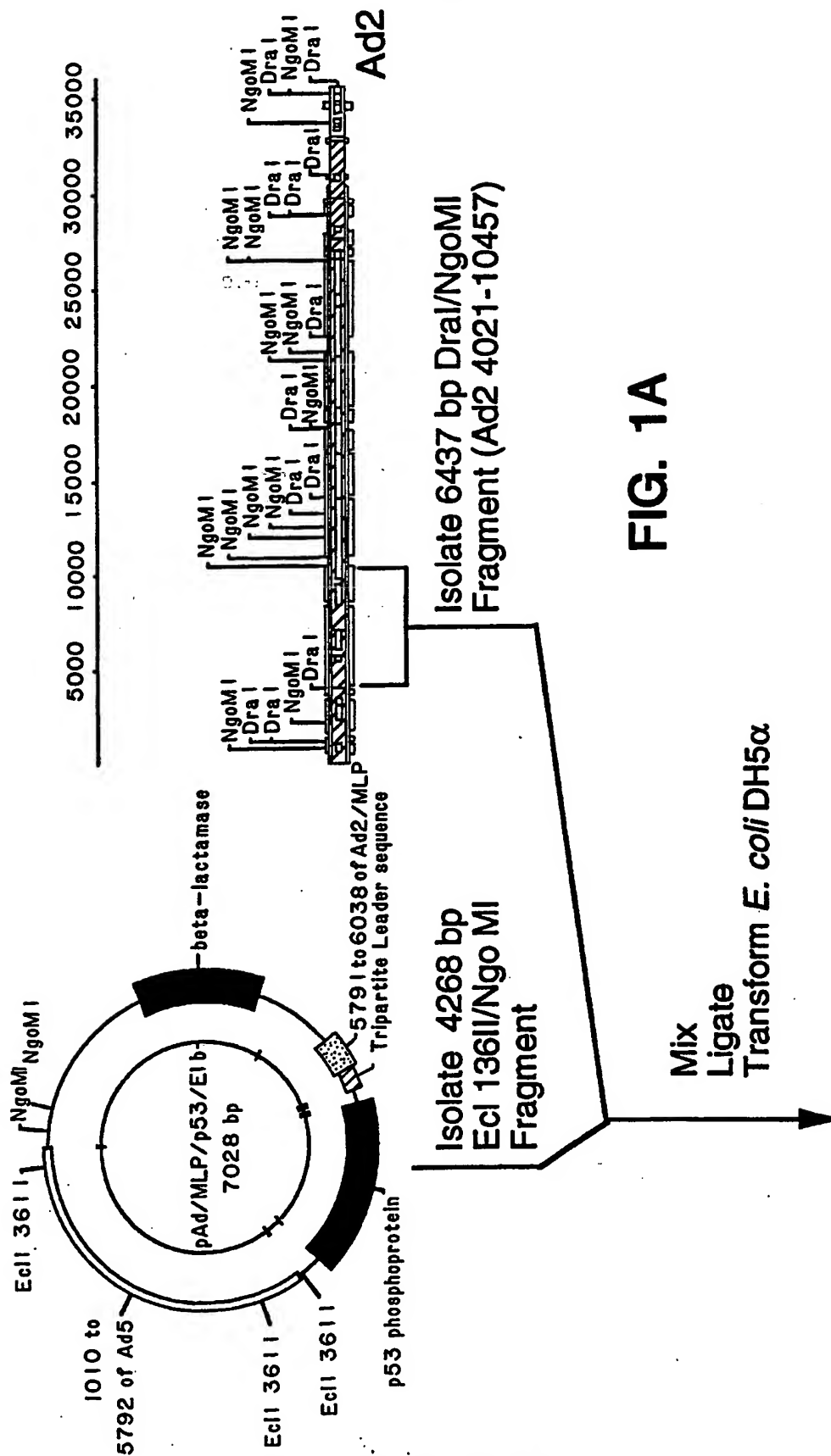


FIG. 1A

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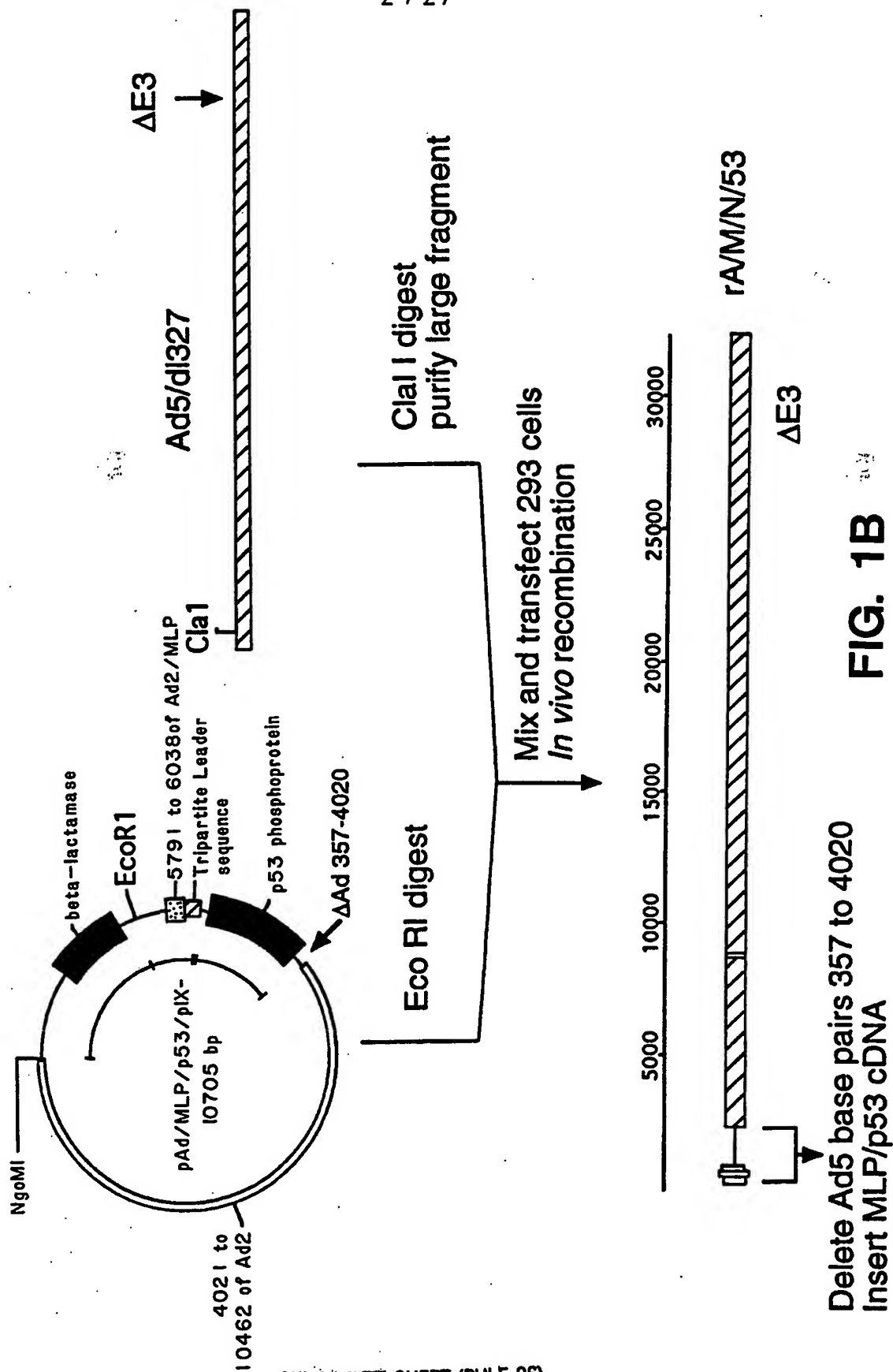


FIG. 1B

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Met Pro Pro Lys Thr Pro Arg Lys Thr Ala Ala Thr Ala Ala Ala Ala
 1 5 10 15
 Ala Ala Glu Pro Pro Ala Pro Pro Pro Pro Pro Pro Pro Glu Glu Asp
 20 25 30
 Pro Glu Gln Asp Ser Gly Pro Glu Asp Leu Pro Leu Val Arg Leu Glu
 35 40 45
 Phe Glu Glu Thr Glu Glu Pro Asp Phe Thr Ala Leu Cys Gln Lys Leu
 50 55 60
 Lys Ile Pro Asp His Val Arg Glu Arg Ala Trp Leu Thr Trp Glu Lys
 65 70 75 80
 Val Ser Ser Val Asp Gly Val Leu Gly Gly Tyr Ile Gln Lys Lys Lys
 85 90 95
 Glu Leu Trp Gly Ile Cys Ile Phe Ile Ala Ala Val Asp Leu Asp Glu
 100 105 110
 Met Ser Phe Thr Phe Thr Glu Leu Gln Lys Asn Ile Glu Ile Ser Val
 115 120 125
 His Lys Phe Phe Asn Leu Leu Lys Glu Ile Asp Thr Ser Thr Lys Val
 130 135 140
 Asp Asn Ala Met Ser Arg Leu Leu Lys Lys Tyr Asp Val Leu Phe Ala
 145 150 155 160
 Leu Phe Ser Lys Leu Glu Arg Thr Cys Glu Leu Ile Tyr Leu Thr Gln
 165 170 175
 Pro Ser Ser Ser Ile Ser Thr Glu Ile Asn Ser Ala Leu Val Leu Lys
 180 185 190
 Val Ser Trp Ile Thr Phe Leu Leu Ala Lys Gly Glu Val Leu Gln Met
 195 200 205
 Glu Asp Asp Leu Val Ile Ser Phe Gln Leu Met Leu Cys Val Leu Asp
 210 215 220
 Tyr Phe Ile Lys Leu Ser Pro Pro Met Leu Leu Lys Glu Pro Tyr Lys
 225 230 235 240
 Thr Ala Val Ile Pro Ile Asn Gly Ser Pro Arg Thr Pro Arg Arg Gly
 245 250 255

FIG. 2A

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Gln Asn Arg Ser Ala Arg Ile Ala Lys Gln Leu Glu Asn Asp Thr Arg
 260 265 270
 Ile Ile Glu Val Leu Cys Lys Glu His Glu Cys Asn Ile Asp Glu Val
 275 280 285
 Lys Asn Val Tyr Phe Lys Asn Phe Ile Pro Phe Met Asn Ser Leu Gly
 290 295 300
 Leu Val Thr Ser Asn Gly Leu Pro Glu Val Glu Asn Leu Ser Lys Arg
 305 310 315 320
 Tyr Glu Glu Ile Tyr Leu Lys Asn Lys Asp Leu Asp Ala Arg Leu Phe
 325 330 335
 Leu Asp His Asp Lys Thr Leu Gln Thr Asp Ser Ile Asp Ser Phe Glu
 340 345 350
 Thr Gln Arg Thr Pro Arg Lys Ser Asn Leu Asp Glu Glu Val Asn Val
 355 360 365
 Ile Pro Pro His Thr Pro Val Arg Thr Val Met Asn Thr Ile Gln Gln
 370 375 380
 Leu Met Met Ile Leu Asn Ser Ala Ser Asp Gln Pro Ser Glu Asn Leu
 385 390 395 400
 Ile Ser Tyr Phe Asn Asn Cys Thr Val Asn Pro Lys Glu Ser Ile Leu
 405 410 415
 Lys Arg Val Lys Asp Ile Gly Tyr Ile Phe Lys Glu Lys Phe Ala Lys
 420 425 430
 Ala Val Gly Gln Gly Cys Val Glu Ile Gly Ser Gln Arg Tyr Lys Leu
 435 440 445
 Gly Val Arg Leu Tyr Tyr Arg Val Met Glu Ser Met Leu Lys Ser Glu
 450 455 460
 Glu Glu Arg Leu Ser Ile Gln Asn Phe Ser Lys Leu Leu Asn Asp Asn
 465 470 475 480
 Ile Phe His Met Ser Leu Leu Ala Cys Ala Leu Glu Val Val Met Ala
 485 490 495
 Thr Tyr Ser Arg Ser Thr Ser Gln Asn Leu Asp Ser Gly Thr Asp Leu
 500 505 510

FIG. 2B

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Ser Phe Pro Trp Ile Leu Asn Val Leu Asn Leu Lys Ala Phe Asp Phe
 515 520 525
 Tyr Lys Val Ile Glu Ser Phe Ile Lys Ala Glu Gly Asn Leu Thr Arg
 530 535 540
 Glu Met Ile Lys His Leu Glu Arg Cys Glu His Arg Ile Met Glu Ser
 545 550 555 560
 Leu Ala Trp Leu Ser Asp Ser Pro Leu Phe Asp Leu Ile Lys Gln Ser
 565 570 575
 Lys Asp Arg Glu Gly Pro Thr Asp His Leu Glu Ser Ala Cys Pro Leu
 580 585 590
 Asn Leu Pro Leu Gln Asn Asn His Thr Ala Ala Asp Met Tyr Leu Ser
 595 600 605
 Pro Val Arg Ser Pro Lys Lys Lys Gly Ser Thr Thr Arg Val Asn Ser
 610 615 620
 Thr Ala Asn Ala Glu Thr Gln Ala Thr Ser Ala Phe Gln Thr Gln Lys
 625 630 635 640
 Pro Leu Lys Ser Thr Ser Leu Ser Leu Phe Tyr Lys Lys Val Tyr Arg
 645 650 655
 Leu Ala Tyr Leu Arg Leu Asn Thr Leu Cys Glu Arg Leu Leu Ser Glu
 660 665 670
 His Pro Glu Leu Glu His Ile Ile Trp Thr Leu Phe Gln His Thr Leu
 675 680 685
 Gln Asn Glu Tyr Glu Leu Met Arg Asp Arg His Leu Asp Gln Ile Met
 690 695 700
 Met Cys Ser Met Tyr Gly Ile Cys Lys Val Lys Asn Ile Asp Leu Lys
 705 710 715 720
 Phe Lys Ile Ile Val Thr Ala Tyr Lys Asp Leu Pro His Ala Val Gln
 725 730 735
 Glu Thr Phe Lys Arg Val Leu Ile Lys Glu Glu Glu Tyr Asp Ser Ile
 740 745 750
 Ile Val Phe Tyr Asn Ser Val Phe Met Gln Arg Leu Lys Thr Asn Ile
 755 760 765

FIG. 2C

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Leu	Gln	Tyr	Ala	Ser	Thr	Arg	Pro	Pro	Thr	Leu	Ser	Pro	Ile	Pro	His
770						775					780				
Ile	Pro	Arg	Ser	Pro	Tyr	Lys	Phe	Pro	Ser	Ser	Pro	Leu	Arg	Ile	Pro
785					790					795					800
Gly	Gly	Asn	Ile	Tyr	Ile	Ser	Pro	Leu	Lys	Ser	Pro	Tyr	Lys	Ile	Ser
				805					810					815	
Glu	Gly	Leu	Pro	Thr	Pro	Thr	Lys	Met	Thr	Pro	Arg	Ser	Arg	Ile	Leu
			820					825					830		
Val	Ser	Ile	Gly	Glu	Ser	Phe	Gly	Thr	Ser	Glu	Lys	Phe	Gln	Lys	Ile
		835					840					845			
Asn	Gln	Met	Val	Cys	Asn	Ser	Asp	Arg	Val	Leu	Lys	Arg	Ser	Ala	Glu
		850				855					860				
Gly	Ser	Asn	Pro	Pro	Lys	Pro	Leu	Lys	Lys	Leu	Arg	Phe	Asp	Ile	Glu
865					870					875					880
Gly	Ser	Asp	Glu	Ala	Asp	Gly	Ser	Lys	His	Leu	Pro	Gly	Glu	Ser	Lys
				885					890					895	
Phe	Gln	Gln	Lys	Leu	Ala	Glu	Met	Thr	Ser	Thr	Arg	Thr	Arg	Met	Gln
			900					905					910		
Lys	Gln	Lys	Met	Asn	Asp	Ser	Met	Asp	Thr	Ser	Asn	Lys	Glu	Glu	Lys
		915					920					925			

FIG. 2D

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TTCCGGTTTT TCTCAGGGGA CGTTGAAATT ATTTTGTAA CGGGAGTCGG GAGAGGACGG																60
GGCGTGCCCC GCGTGCGCGC GCGTCGTCCT CCCCGGCGCT CCTCCACAGC TCGCTGGCTC																120
CCGCCGCGGA AAGGCGTC ATG CCG CCC AAA ACC CCC CGA AAA ACG GCC GCC																171
Met Pro Pro Lys Thr Pro Arg Lys Thr Ala Ala																
1 5 10																
ACC GCC GCC GCT GCC GCC GCG GAA CCC CCG GCA CCG CCG CCG CCG CCC																219
Thr Ala Ala Ala Ala Ala Ala Glu Pro Pro Ala Pro Pro Pro Pro Pro																
15 20 25																
CCT CCT GAG GAG GAC CCA GAG CAG GAC AGC GGC CCG GAG GAC CTG CCT																267
Pro Pro Glu Glu Asp, Pro Glu Gln Asp Ser Gly Pro Glu Asp Leu Pro																
30 35 40																
CTC GTC AGG CTT GAG TTT GAA GAA ACA GAA GAA CCT GAT TTT ACT GCA																315
Leu Val Arg Leu Glu Phe Glu Glu Thr Glu Glu Pro Asp Phe Thr Ala																
45 50 55																
TTA TGT CAG AAA TTA AAG ATA CCA GAT CAT GTC AGA GAG AGA GCT TGG																363
Leu Cys Gln Lys Leu Lys Ile Pro Asp His Val Arg Glu Arg Ala Trp																
60 65 70 75																
TTA ACT TGG GAG AAA GTT TCA TCT GTG GAT GGA GTA TTG GGA GGT TAT																411
Leu Thr Trp Glu Lys Val Ser Ser Val Asp Gly Val Leu Gly Gly Tyr																
80 85 90																
ATT CAA AAG AAA AAG GAA CTG TGG GGA ATC TGT ATC TTT ATT GCA GCA																459
Ile Gln Lys Lys Lys Glu Leu Trp Gly Ile Cys Ile Phe Ile Ala Ala																
95 100 105																
GTT GAC CTA GAT GAG ATG TCG TTC ACT TTT ACT GAG CTA CAG AAA AAC																507
Val Asp Leu Asp Glu Met Ser Phe Thr Phe Thr Glu Leu Gln Lys Asn																
110 115 120																
ATA GAA ATC AGT GTC CAT AAA TTC TTT AAC TTA CTA AAA GAA ATT GAT																555
Ile Glu Ile Ser Val His Lys Phe Phe Asn Leu Leu Lys Glu Ile Asp																
125 130 135																
ACC AGT ACC AAA GTT GAT AAT GCT ATG TCA AGA CTG TTG AAG AAG TAT																603
Thr Ser Thr Lys Val Asp Asn Ala Met Ser Arg Leu Leu Lys Lys Tyr																
140 145 150 155																
GAT GTA TTG TTT GCA CTC TTC AGC AAA TTG GAA AGG ACA TGT GAA CTT																651
Asp Val Leu Phe Ala Leu Phe Ser Lys Leu Glu Arg Thr Cys Glu Leu																
160 165 170																
ATA TAT TTG ACA CAA CCC AGC AGT TCG ATA TCT ACT GAA ATA AAT TCT																699
Ile Tyr Leu Thr Gln Pro Ser Ser Ser Ile Ser Thr Glu Ile Asn Ser																
175 180 185																
GCA TTG GTG CTA AAA GTT TCT TGG ATC ACA TTT TTA TTA GCT AAA GGG																747
Ala Leu Val Leu Lys Val Ser Trp Ile Thr Phe Leu Leu Ala Lys Gly																
190 195 200																
GAA GTA TTA CAA ATG GAA GAT GAT CTG GTG ATT TCA TTT CAG TTA ATG																795
Glu Val Leu Gln Met Glu Asp Asp Leu Val Ile Ser Phe Gln Leu Met																
205 210 215																

FIG. 3A
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CTA	TGT	GTC	CTT	GAC	TAT	TTT	ATT	AAA	CTC	TCA	CCT	CCC	ATG	TTG	CTC	843
Leu	Cys	Val	Leu	Asp	Tyr	Phe	Ile	Lys	Leu	Ser	Pro	Pro	Met	Leu	Leu	
220					225					230					235	
AAA	GAA	CCA	TAT	AAA	ACA	GCT	GTT	ATA	CCC	ATT	AAT	GGT	TCA	CCT	CGA	891
Lys	Glu	Pro	Tyr	Lys	Thr	Ala	Val	Ile	Pro	Ile	Asn	Gly	Ser	Pro	Arg	
				240					245						250	
ACA	CCC	AGG	CGA	GGT	CAG	AAC	AGG	AGT	GCA	CGG	ATA	GCA	AAA	CAA	CTA	939
Thr	Pro	Arg	Arg	Gly	Gln	Asn	Arg	Ser	Ala	Arg	Ile	Ala	Lys	Gln	Leu	
			255					260							265	
GAA	AAT	GAT	ACA	AGA	ATT	ATT	GAA	GTT	CTC	TGT	AAA	GAA	CAT	GAA	TGT	987
Glu	Asn	Asp	Thr	Arg	Ile	Ile	Glu	Val	Leu	Cys	Lys	Glu	His	Glu	Cys	
		270					275								280	
AAT	ATA	GAT	GAG	GTG	AAA	AAT	GTT	TAT	TTC	AAA	AAT	TTT	ATA	CCT	TTT	1035
Asn	Ile	Asp	Glu	Val	Lys	Asn	Val	Tyr	Phe	Lys	Asn	Phe	Ile	Pro	Phe	
	285					290									295	
ATG	AAT	TCT	CTT	GGA	CTT	GTA	ACA	TCT	AAT	GGA	CTT	CCA	GAG	GTT	GAA	1083
Met	Asn	Ser	Leu	Gly	Leu	Val	Thr	Ser	Asn	Gly	Leu	Pro	Glu	Val	Glu	
300					305					310					315	
AAT	CTT	TCT	AAA	CGA	TAC	GAA	GAA	ATT	TAT	CTT	AAA	AAT	AAA	GAT	CTA	1131
Asn	Leu	Ser	Lys	Arg	Tyr	Glu	Glu	Ile	Tyr	Leu	Lys	Asn	Lys	Asp	Leu	
				320					325					330		
GAT	GCA	AGA	TTA	TTT	TTG	GAT	CAT	GAT	AAA	ACT	CTT	CAG	ACT	GAT	TCT	1179
Asp	Ala	Arg	Leu	Phe	Leu	Asp	His	Asp	Lys	Thr	Leu	Gln	Thr	Asp	Ser	
			335					340						345		
ATA	GAC	AGT	TTT	GAA	ACA	CAG	AGA	ACA	CCA	CGA	AAA	AGT	AAC	CTT	GAT	1227
Ile	Asp	Ser	Phe	Glu	Thr	Gln	Arg	Thr	Pro	Arg	Lys	Ser	Asn	Leu	Asp	
			350				355							360		
GAA	GAG	GTG	AAT	GTA	ATT	CCT	CCA	CAC	ACT	CCA	GTT	AGG	ACT	GTT	ATG	1275
Glu	Glu	Val	Asn	Val	Ile	Pro	Pro	His	Thr	Pro	Val	Arg	Thr	Val	Met	
	365					370					375					
AAC	ACT	ATC	CAA	CAA	TTA	ATG	ATG	ATT	TTA	AAT	TCA	GCA	AGT	GAT	CAA	1323
Asn	Thr	Ile	Gln	Gln	Leu	Met	Met	Ile	Leu	Asn	Ser	Ala	Ser	Asp	Gln	
380					385					390					395	
CCT	TCA	GAA	AAT	CTG	ATT	TCC	TAT	TTT	AAC	AAC	TGC	ACA	GTG	AAT	CCA	1371
Pro	Ser	Glu	Asn	Leu	Ile	Ser	Tyr	Phe	Asn	Asn	Cys	Thr	Val	Asn	Pro	
				400					405					410		
AAA	GAA	AGT	ATA	CTG	AAA	AGA	GTG	AAG	GAT	ATA	GGA	TAC	ATC	TTT	AAA	1419
Lys	Glu	Ser	Ile	Leu	Lys	Arg	Val	Lys	Asp	Ile	Gly	Tyr	Ile	Phe	Lys	
			415					420						425		
GAG	AAA	TTT	GCT	AAA	GCT	GTG	GGA	CAG	GGT	TGT	GTC	GAA	ATT	GGA	TCA	1467
Glu	Lys	Phe	Ala	Lys	Ala	Val	Gly	Gln	Gly	Cys	Val	Glu	Ile	Gly	Ser	
		430					435					440				
CAG	CGA	TAC	AAA	CTT	GGA	GTT	CGC	TTG	TAT	TAC	CGA	GTA	ATG	GAA	TCC	1515
Gln	Arg	Tyr	Lys	Leu	Gly	Val	Arg	Leu	Tyr	Tyr	Arg	Val	Met	Glu	Ser	
	445					450					455					

FIG. 3B

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ATG CTT AAA TCA GAA GAA GAA CGA TTA TCC ATT CAA AAT TTT AGC AAA	1563
Met Leu Lys Ser Glu Glu Glu Arg Leu Ser Ile Gln Asn Phe Ser Lys	
460 465 470 475	
CTT CTG AAT GAC AAC ATT TTT CAT ATG TCT TTA TTG GCG TGC GCT CTT	1611
Leu Leu Asn Asp Asn Ile Phe His Met Ser Leu Leu Ala Cys Ala Leu	
480 485 490	
GAG GTT GTA ATG GCC ACA TAT AGC AGA AGT ACA TCT CAG AAT CTT GAT	1659
Glu Val Val Met Ala Thr Tyr Ser Arg Ser Thr Ser Gln Asn Leu Asp	
495 500 505	
TCT GGA ACA GAT TTG TCT TTC CCA TGG ATT CTG AAT GTG CTT AAT TTA	1707
Ser Gly Thr Asp Leu Ser Phe Pro Trp Ile Leu Asn Val Leu Asn Leu	
510 515 520	
AAA GCC TTT GAT TTT TAC AAA GTG ATC GAA AGT TTT ATC AAA GCA GAA	1755
Lys Ala Phe Asp Phe Tyr Lys Val Ile Glu Ser Phe Ile Lys Ala Glu	
525 530 535	
GGC AAC TTG ACA AGA GAA ATG ATA AAA CAT TTA GAA CGA TGT GAA CAT	1803
Gly Asn Leu Thr Arg Glu Met Ile Lys His Leu Glu Arg Cys Glu His	
540 545 550 555	
CGA ATC ATG GAA TCC CTT GCA TGG CTC TCA GAT TCA CCT TTA TTT GAT	1851
Arg Ile Met Glu Ser Leu Ala Trp Leu Ser Asp Ser Pro Leu Phe Asp	
560 565 570	
CTT ATT AAA CAA TCA AAG GAC CGA GAA GGA CCA ACT GAT CAC CTT GAA	1899
Leu Ile Lys Gln Ser Lys Asp Arg Glu Gly Pro Thr Asp His Leu Glu	
575 580 585	
TCT GCT TGT CCT CTT AAT CTT CCT CTC CAG AAT AAT CAC ACT GCA GCA	1947
Ser Ala Cys Pro Leu Asn Leu Pro Leu Gln Asn Asn His Thr Ala Ala	
590 595 600	
GAT ATG TAT CTT TCT CCT GTA AGA TCT CCA AAG AAA AAA GGT TCA ACT	1995
Asp Met Tyr Leu Ser Pro Val Arg Ser Pro Lys Lys Lys Gly Ser Thr	
605 610 615	
ACG CGT GTA AAT TCT ACT GCA AAT GCA GAG ACA CAA GCA ACC TCA GCC	2043
Thr Arg Val Asn Ser Thr Ala Asn Ala Glu Thr Gln Ala Thr Ser Ala	
620 625 630 635	
TTC CAG ACC CAG AAG CCA TTG AAA TCT ACC TCT CTT TCA CTG TTT TAT	2091
Phe Gln Thr Gln Lys Pro Leu Lys Ser Thr Ser Leu Ser Leu Phe Tyr	
640 645 650	
AAA AAA GTG TAT CGG CTA GCC TAT CTC CGG CTA AAT ACA CTT TGT GAA	2139
Lys Lys Val Tyr Arg Leu Ala Tyr Leu Arg Leu Asn Thr Leu Cys Glu	
655 660 665	
CGC CTT CTG TCT GAG CAC CCA GAA TTA GAA CAT ATC ATC TGG ACC CTT	2187
Arg Leu Leu Ser Glu His Pro Glu Leu Glu His Ile Ile Trp Thr Leu	
670 675 680	
TTC CAG CAC ACC CTG CAG AAT GAG TAT GAA CTC ATG AGA GAC AGG CAT	2235
Phe Gln His Thr Leu Gln Asn Glu Tyr Glu Leu Met Arg Asp Arg His	
685 690 695	

FIG. 3C

SUBSTITUTE SHEET (RULE 26)

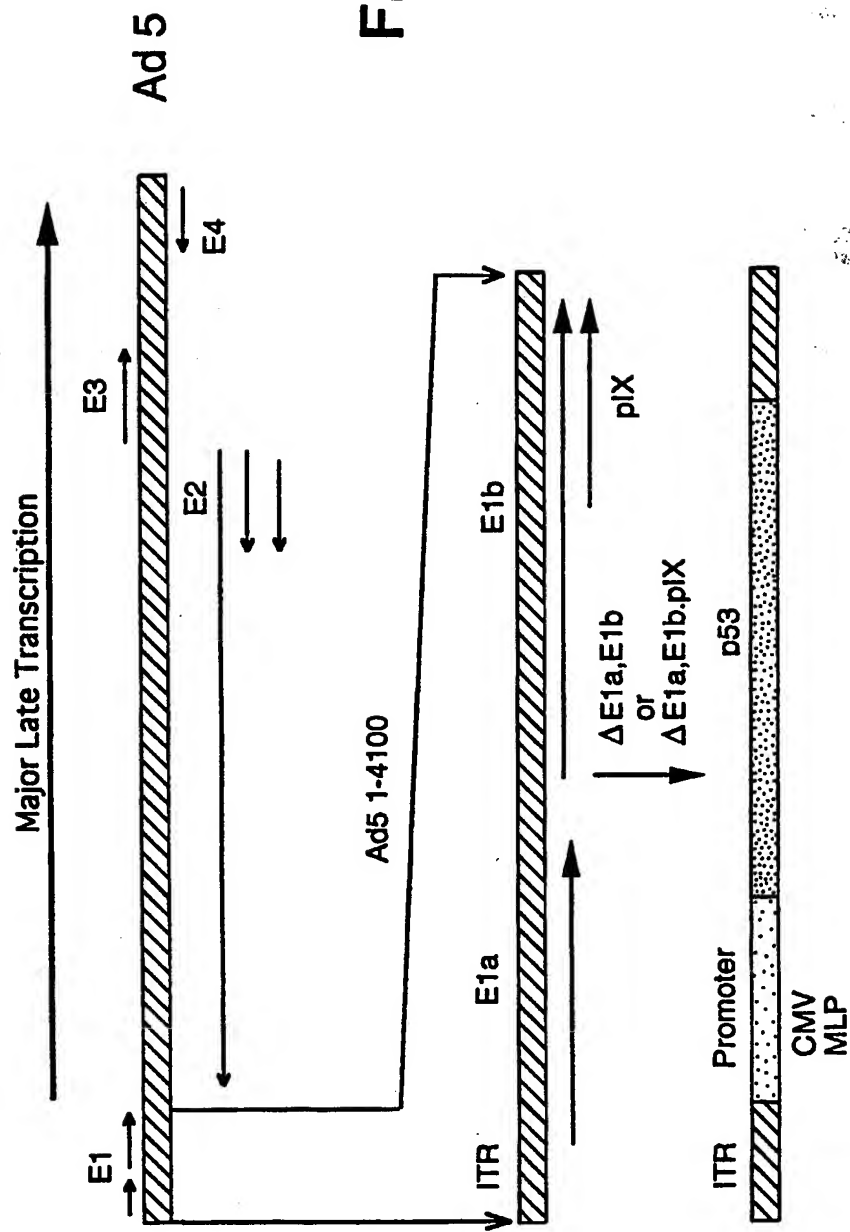
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TTG	GAC	CAA	ATT	ATG	ATG	TGT	TCC	ATG	TAT	GGC	ATA	TGC	AAA	GTG	AAG	2283
Leu	Asp	Gln	Ile	Met	Met	Cys	Ser	Met	Tyr	Gly	Ile	Cys	Lys	Val	Lys	
700					705					710					715	
AAT	ATA	GAC	CTT	AAA	TTC	AAA	ATC	ATT	GTA	ACA	GCA	TAC	AAG	GAT	CTT	2331
Asn	Ile	Asp	Leu	Lys	Phe	Lys	Ile	Ile	Val	Thr	Ala	Tyr	Lys	Asp	Leu	
				720					725						730	
CCT	CAT	GCT	GTT	CAG	GAG	ACA	TTC	AAA	CGT	GTT	TTG	ATC	AAA	GAA	GAG	2379
Pro	His	Ala	Val	Gln	Glu	Thr	Phe	Lys	Arg	Val	Leu	Ile	Lys	Glu	Glu	
			735					740					745			
GAG	TAT	GAT	TCT	ATT	ATA	GTA	TTC	TAT	AAC	TCG	GTC	TTC	ATG	CAG	AGA	2427
Glu	Tyr	Asp	Ser	Ile	Ile	Val	Phe	Tyr	Asn	Ser	Val	Phe	Met	Gln	Arg	
		750					755					760				
CTG	AAA	ACA	AAT	ATT	TTG	CAG	TAT	GCT	TCC	ACC	AGG	CCC	CCT	ACC	TTG	2475
Leu	Lys	Thr	Asn	Ile	Leu	Gln	Tyr	Ala	Ser	Thr	Arg	Pro	Pro	Thr	Leu	
	765					770					775					
TCA	CCA	ATA	CCT	CAC	ATT	CCT	CGA	AGC	CCT	TAC	AAG	TTT	CCT	AGT	TCA	2523
Ser	Pro	Ile	Pro	His	Ile	Pro	Arg	Ser	Pro	Tyr	Lys	Phe	Pro	Ser	Ser	
780					785					790					795	
CCC	TTA	CGG	ATT	CCT	GGA	GGG	AAC	ATC	TAT	ATT	TCA	CCC	CTG	AAG	AGT	2571
Pro	Leu	Arg	Ile	Pro	Gly	Gly	Asn	Ile	Tyr	Ile	Ser	Pro	Leu	Lys	Ser	
				800					805					810		
CCA	TAT	AAA	ATT	TCA	GAA	GGT	CTG	CCA	ACA	CCA	ACA	AAA	ATG	ACT	CCA	2619
Pro	Tyr	Lys	Ile	Ser	Glu	Gly	Leu	Pro	Thr	Pro	Thr	Lys	Met	Thr	Pro	
			815					820					825			
AGA	TCA	AGA	ATC	TTA	GTA	TCA	ATT	GGT	GAA	TCA	TTC	GGG	ACT	TCT	GAG	2667
Arg	Ser	Arg	Ile	Leu	Val	Ser	Ile	Gly	Glu	Ser	Phe	Gly	Thr	Ser	Glu	
		830					835					840				
AAG	TTC	CAG	AAA	ATA	AAT	CAG	ATG	GTA	TGT	AAC	AGC	GAC	CGT	GTG	CTC	2715
Lys	Phe	Gln	Lys	Ile	Asn	Gln	Met	Val	Cys	Asn	Ser	Asp	Arg	Val	Leu	
	845					850					855					
AAA	AGA	AGT	GCT	GAA	GGA	AGC	AAC	CCT	CCT	AAA	CCA	CTG	AAA	AAA	CTA	2763
Lys	Arg	Ser	Ala	Glu	Gly	Ser	Asn	Pro	Pro	Lys	Pro	Leu	Lys	Lys	Leu	
860					865					870					875	
CGC	TTT	GAT	ATT	GAA	GGA	TCA	GAT	GAA	GCA	GAT	GGA	AGT	AAA	CAT	CTC	2811
Arg	Phe	Asp	Ile	Glu	Gly	Ser	Asp	Glu	Ala	Asp	Gly	Ser	Lys	His	Leu	
				880				885						890		
CCA	GGA	GAG	TCC	AAA	TTT	CAG	CAG	AAA	CTG	GCA	GAA	ATG	ACT	TCT	ACT	2859
Pro	Gly	Glu	Ser	Lys	Phe	Gln	Gln	Lys	Leu	Ala	Glu	Met	Thr	Ser	Thr	
			895					900					905			
CGA	ACA	CGA	ATG	CAA	AAG	CAG	AAA	ATG	AAT	GAT	AGC	ATG	GAT	ACC	TCA	2907
Arg	Thr	Arg	Met	Gln	Lys	Gln	Lys	Met	Asn	Asp	Ser	Met	Asp	Thr	Ser	
		910				915						920				
AAC	AAG	GAA	GAG	AAA	TGAGGATCTC	AGGACCTTGG	TGGACACTGT	GTACACCTCT								2962
Asn	Lys	Glu	Glu	Lys												
		925														
GGATTTCATTG	TCTCTCACAG	ATGTGACTGA	TAT													2995

FIG. 3D
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FIG. 4



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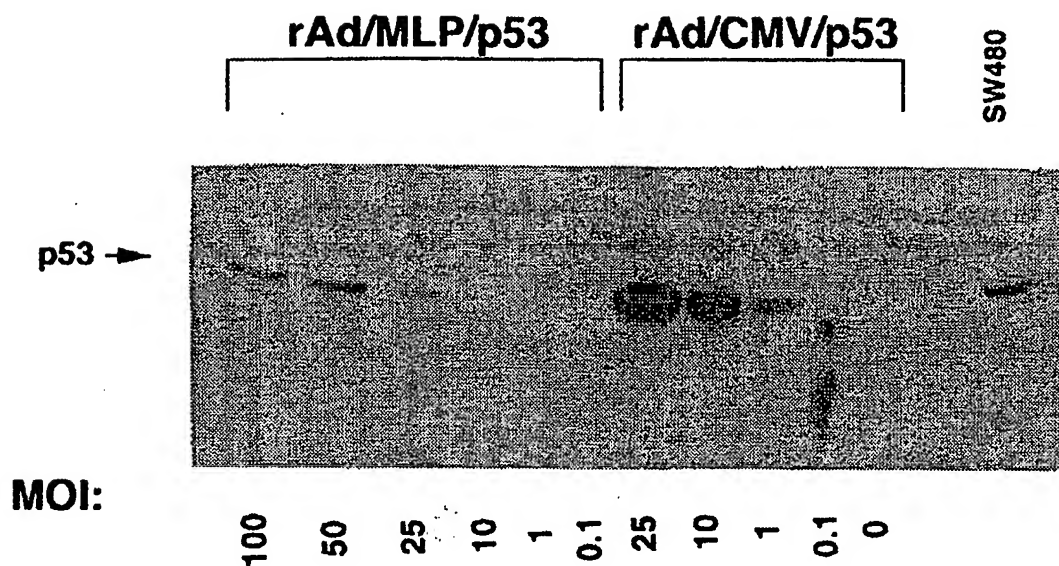


FIG. 5A

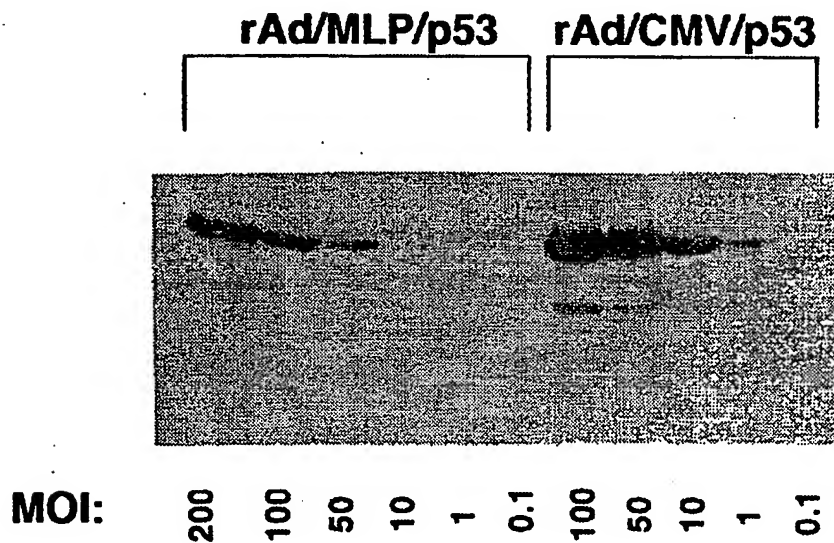


FIG. 5B

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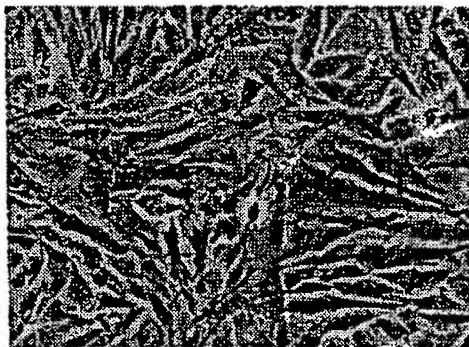


FIG. 6A

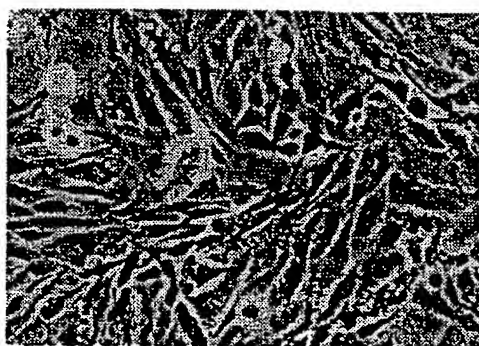
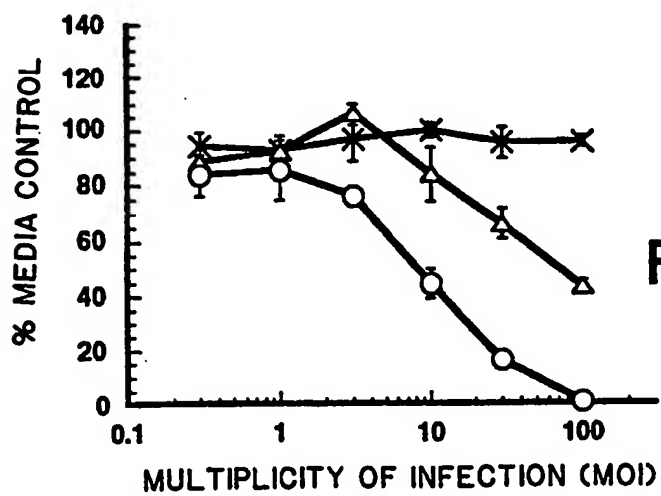
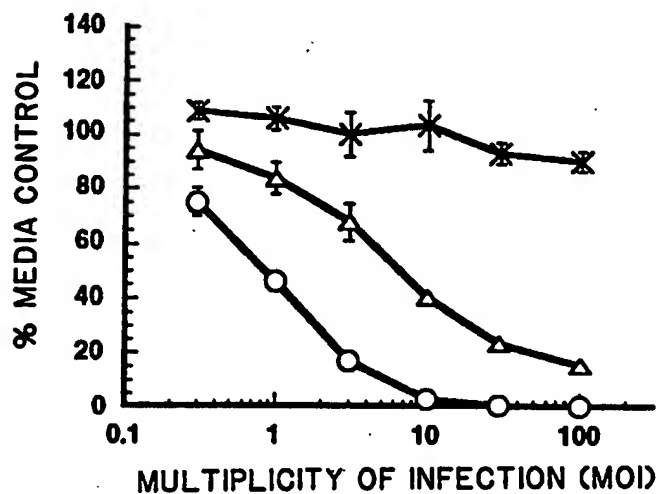
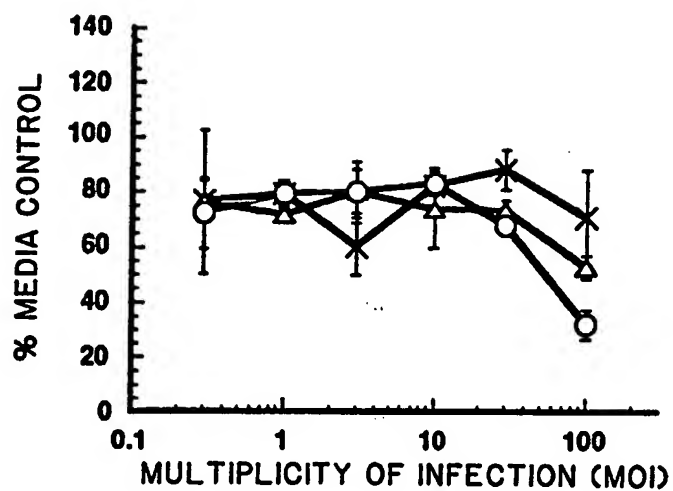


FIG. 6B



FIG. 6C

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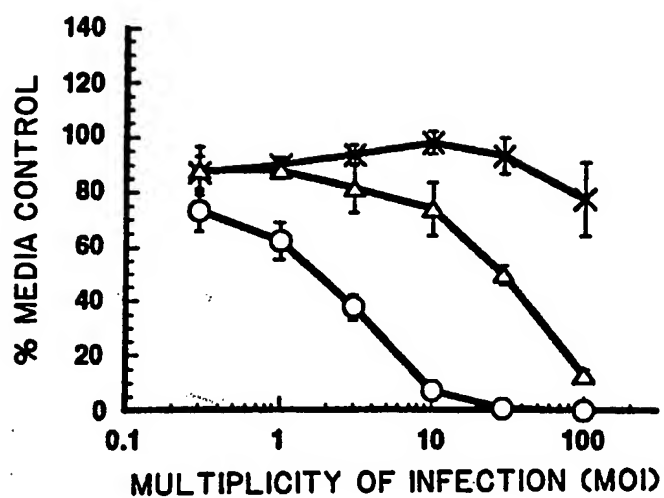


FIG. 7D

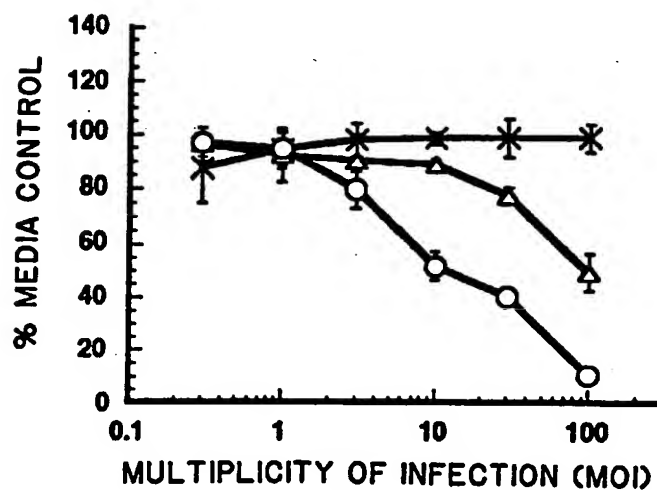


FIG. 7E

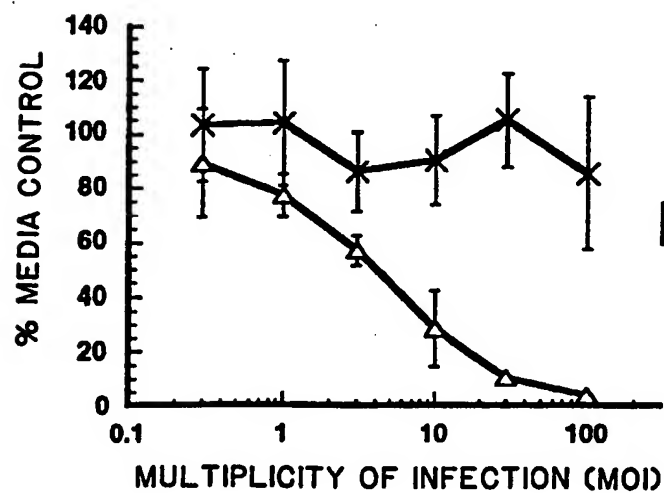
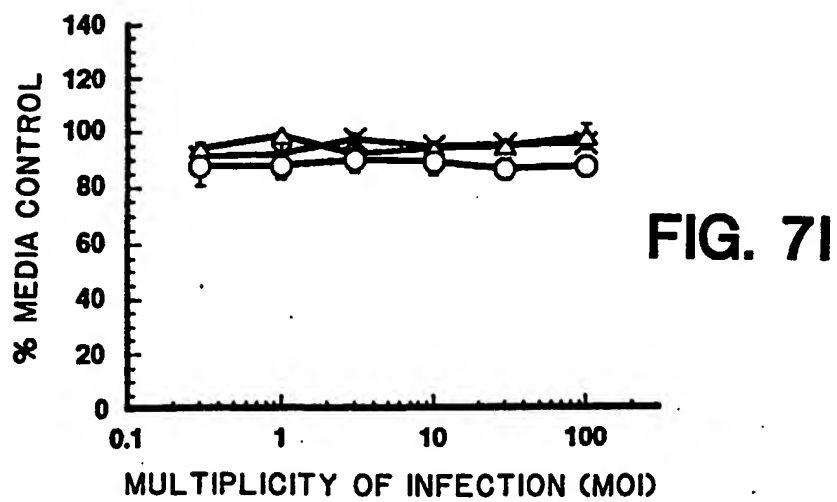
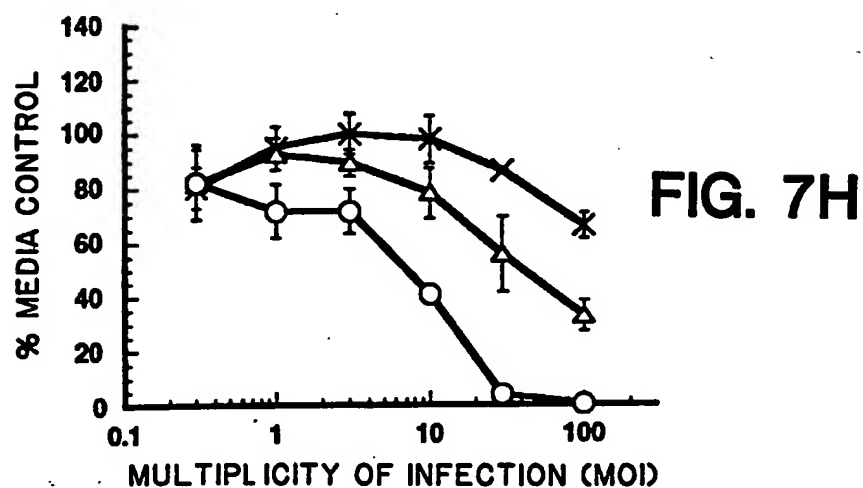
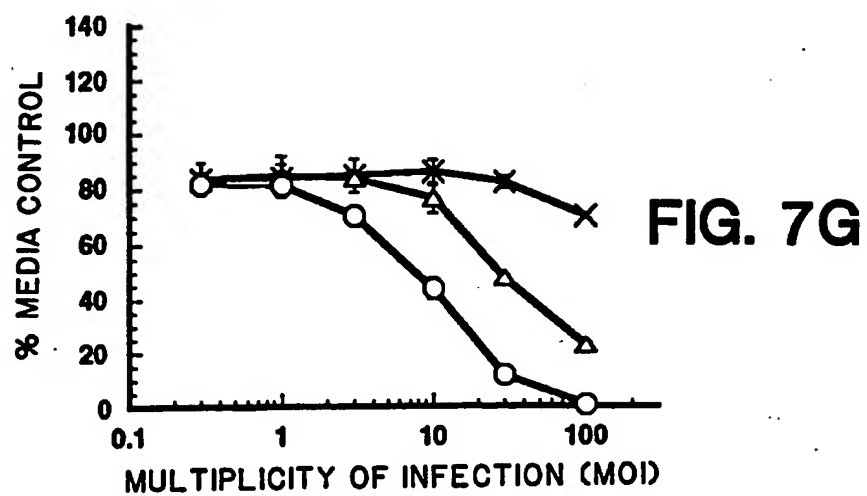
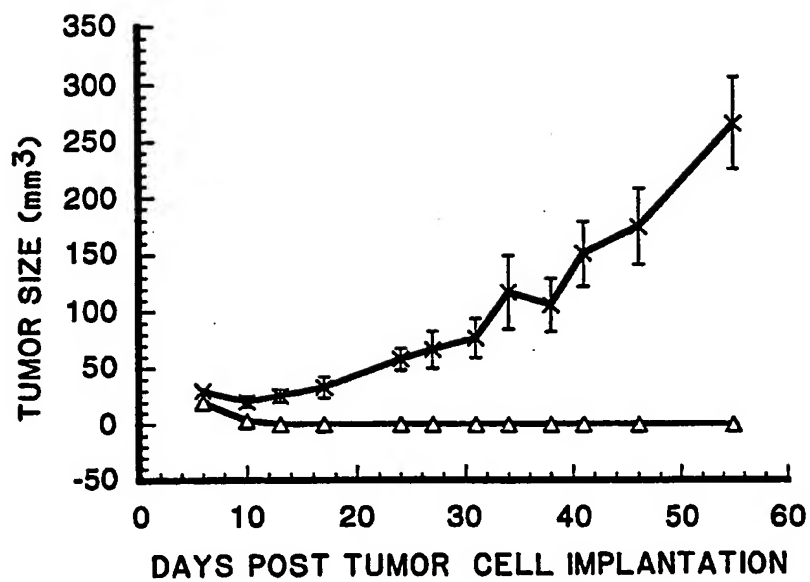


FIG. 7F

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**FIG. 8**

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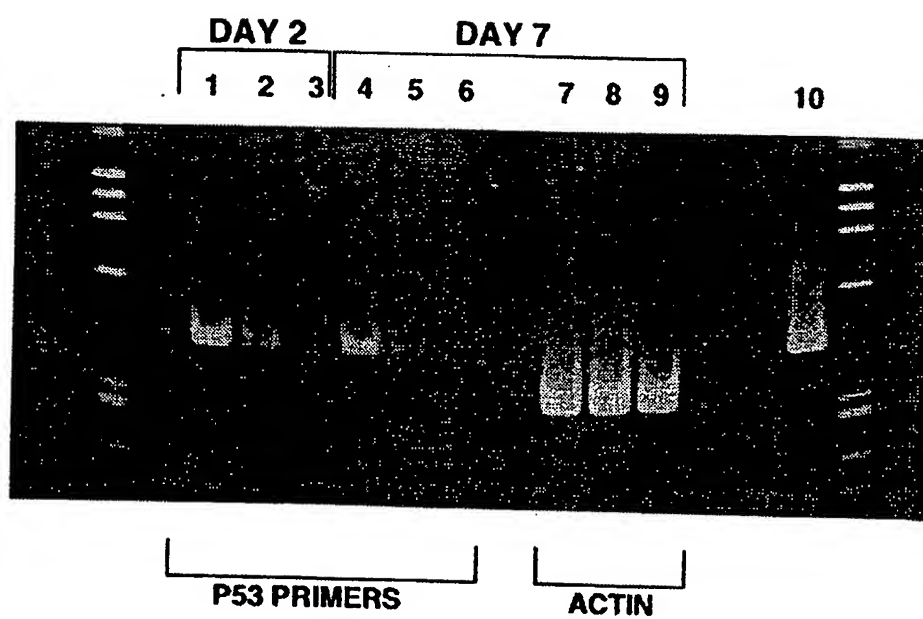


FIG. 9

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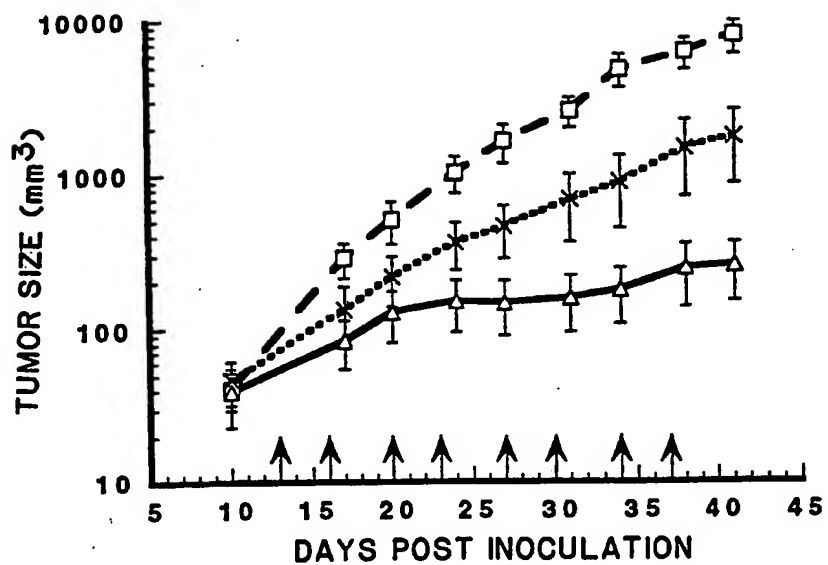


FIG. 10A

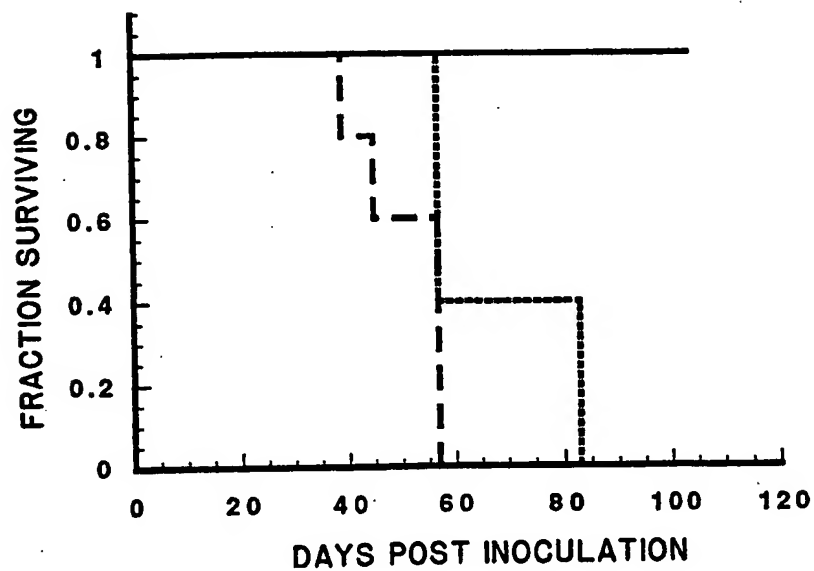


FIG. 10B

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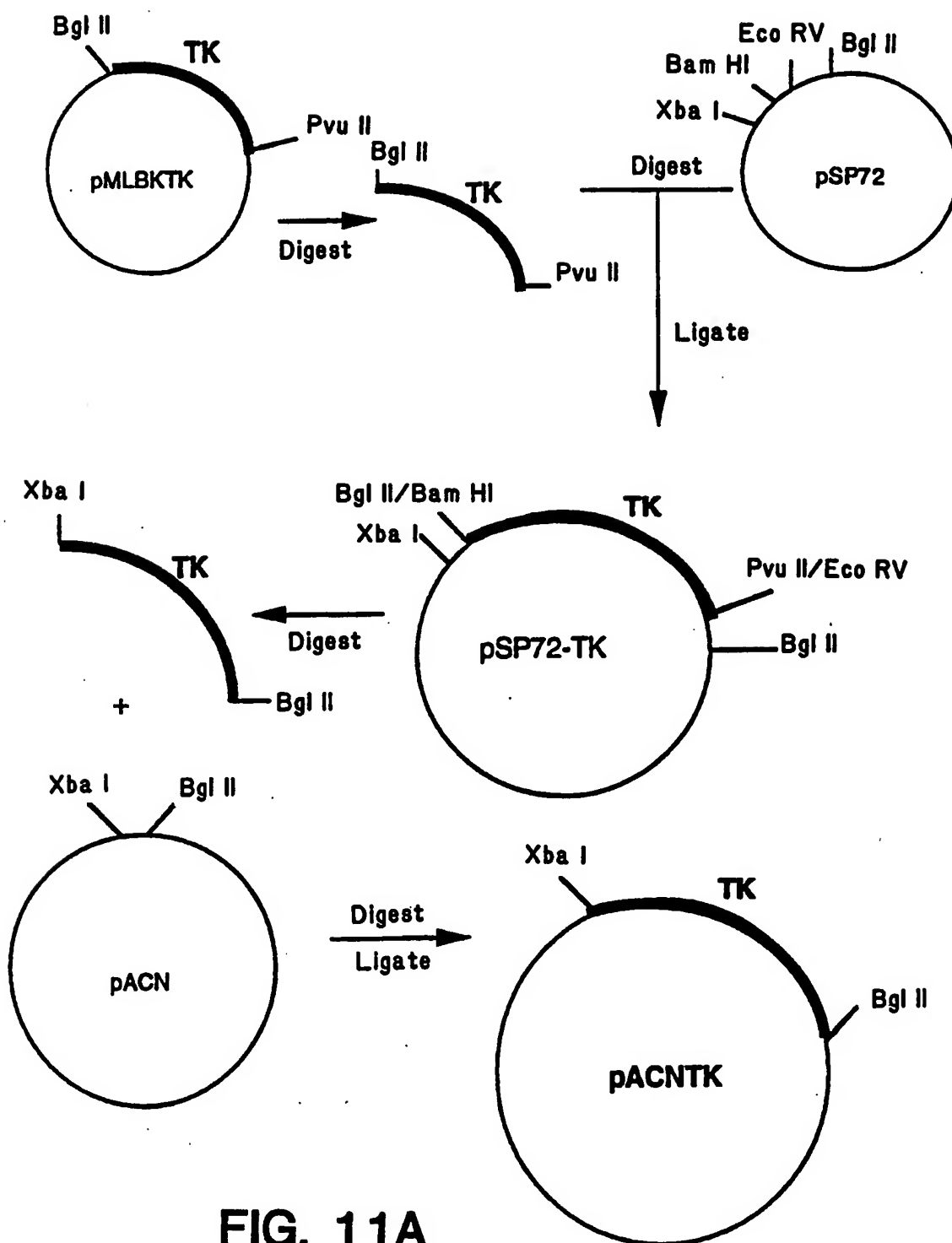
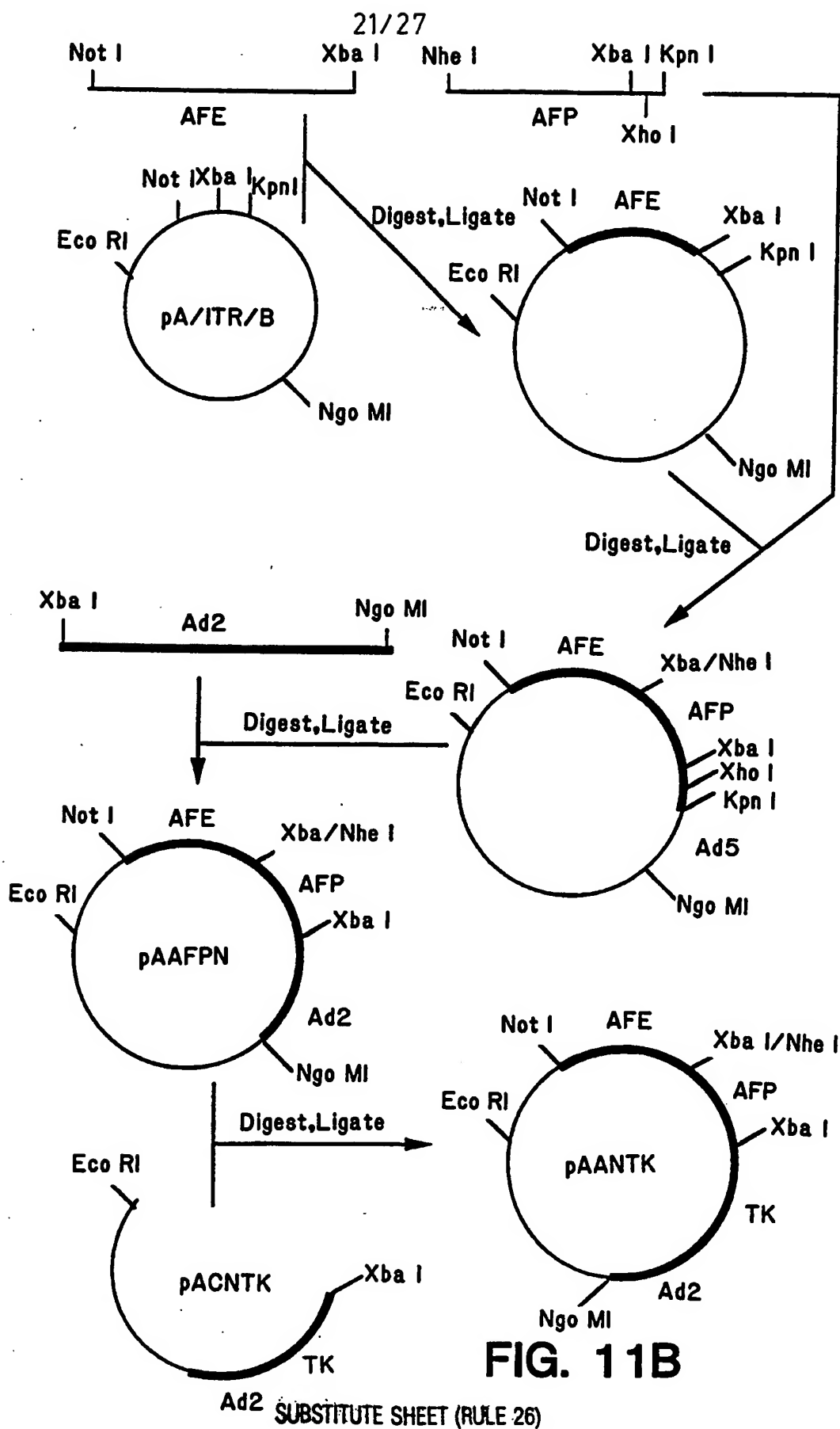


FIG. 11A



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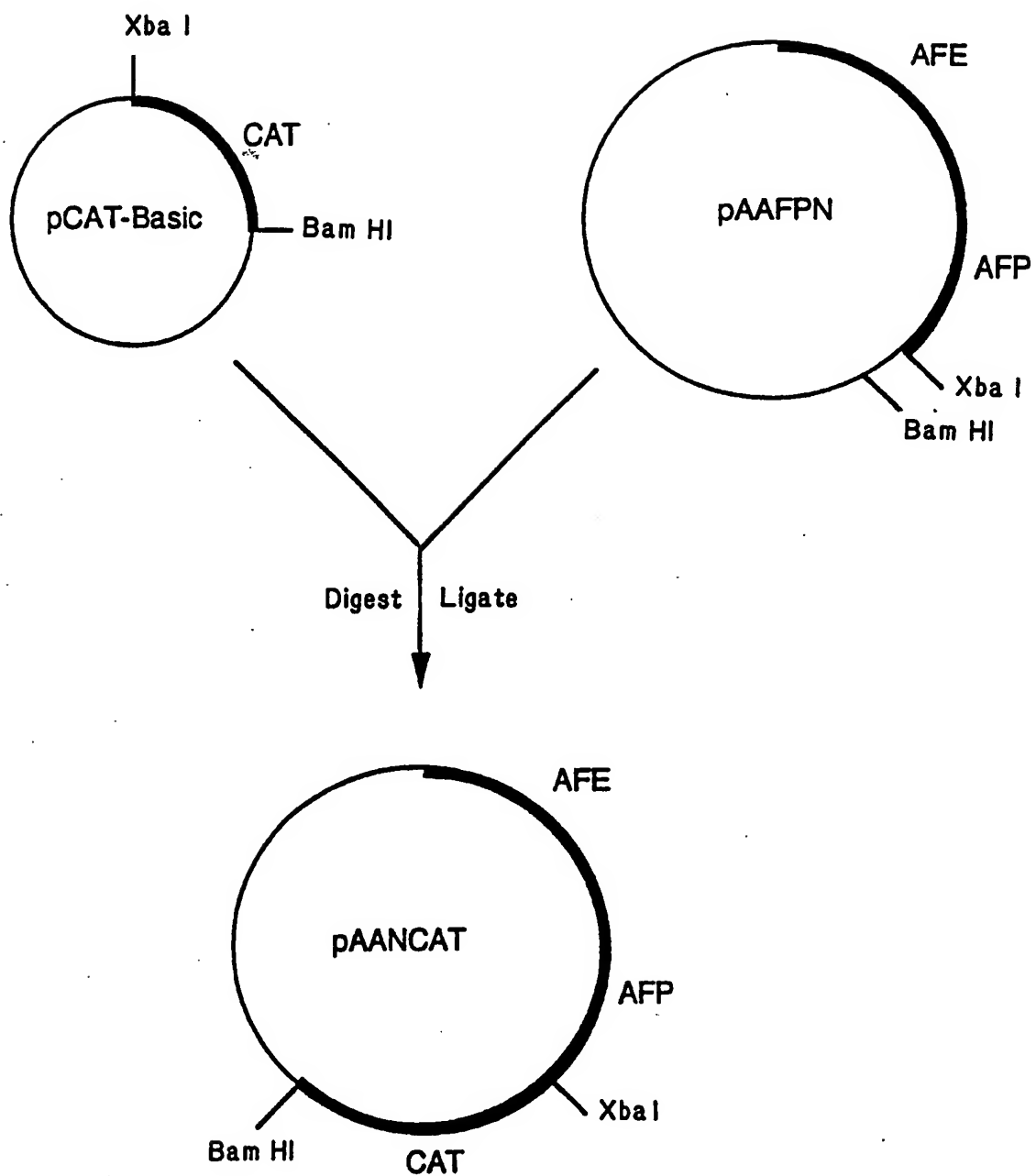


FIG. 11C

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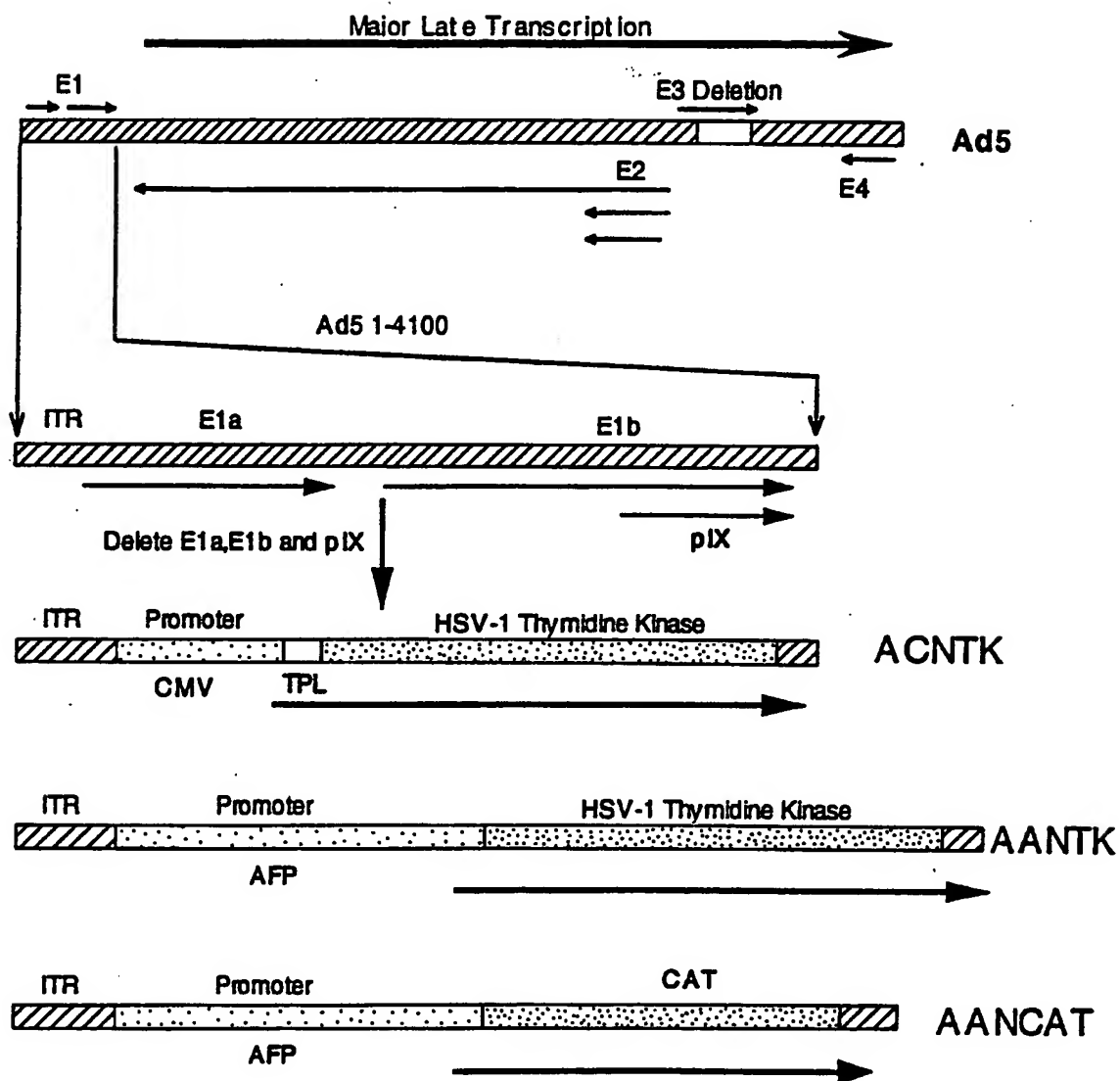


FIG. 12

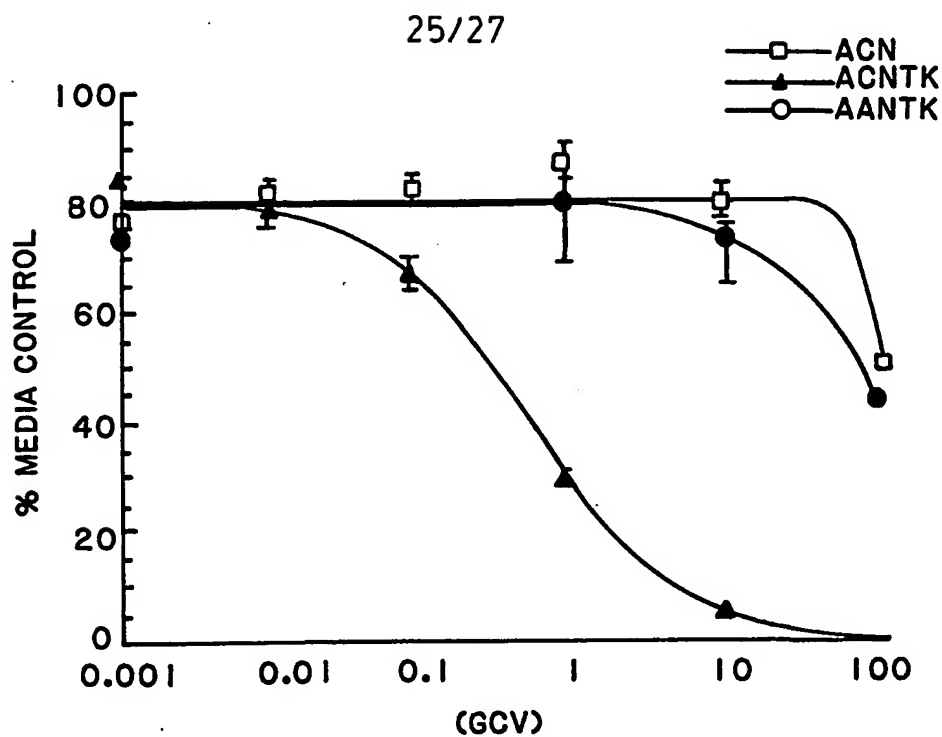


FIG. 14A

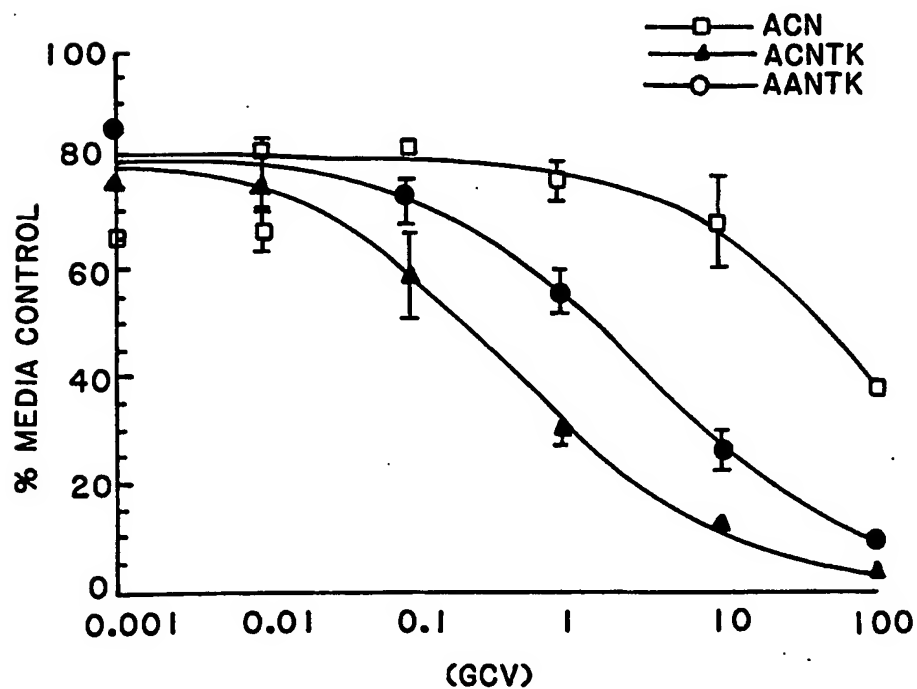


FIG. 14B

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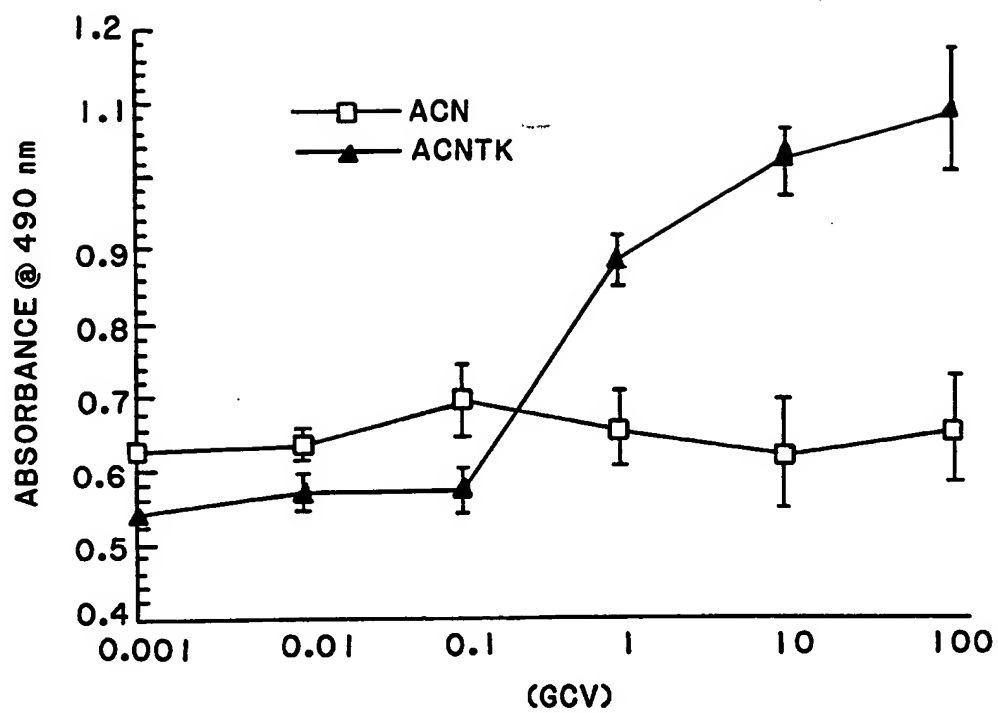


FIG. 15

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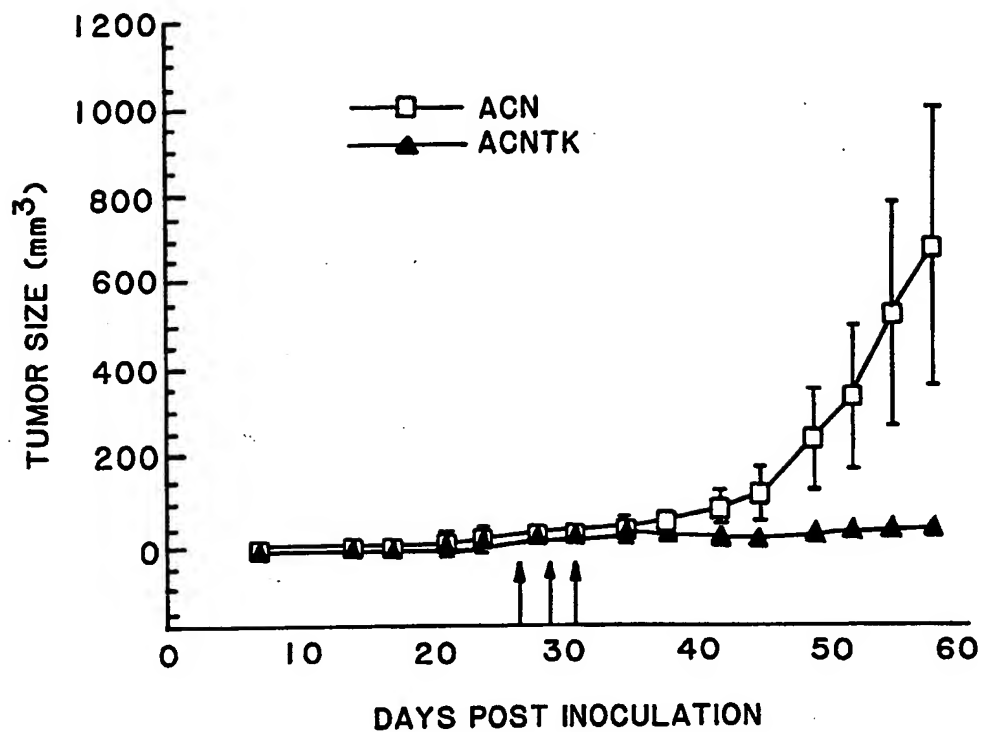


FIG. 16A

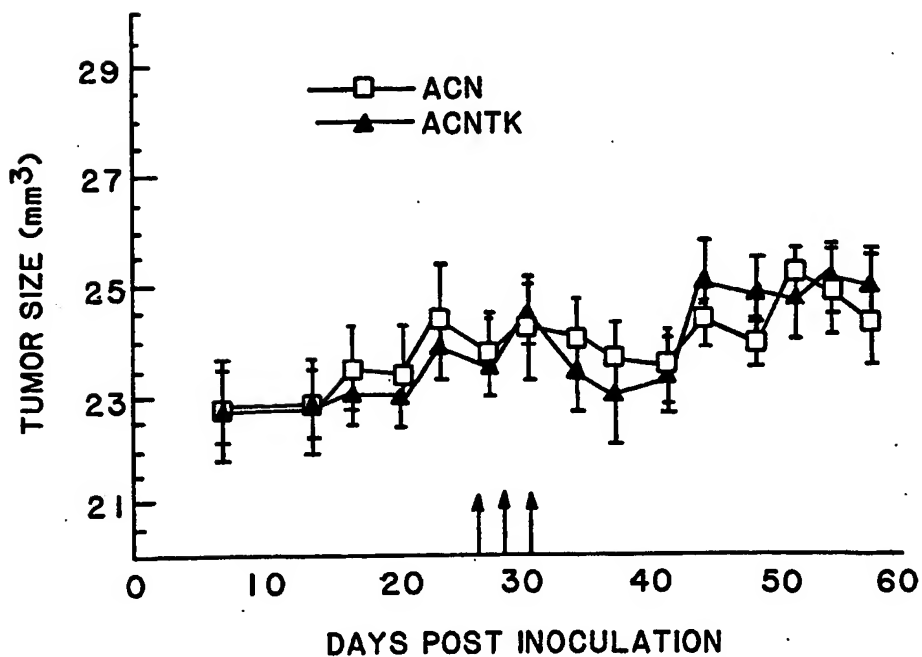


FIG. 16B